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The role of the homeodomain protein Bozozok in Zebrafish axis formation

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ABSTRACT The zebrafish *bozozok* (*boz*) gene encoding a homeodomain protein (also named Dharma/Nieuwkoid) is required during blastula stages for the formation of a complete Spemann-Mangold gastrula organizer and subsequent development of axial mesoderm and anterior neural structures. Expression of *boz* in the dorsal yolk syncytial layer (YSL) and overlying marginal blastomeres is activated by β-catenin. Bozozok itself acts as a transcriptional repressor, and promotes organizer formation by directly inhibiting expression of the *bmp2b* (*swirl)* gene and by negatively regulating Wnt signaling by an unknown mechanism. *boz* cooperates with the Nodal-related secreted factors, Cyclops and Squint, in organizer formation. The incomplete organizer in *boz* mutants is deficient in expression of a number of factors such as Chordin that antagonize Bone morphogenetic proteins (Bmps), and Dickkopf 1, a Wnt antagonist. Conversely, the dorsal blastoderm of *boz* mutants exhibits ectopic expression of genes normally excluded from the dorsal midline such as *wnt8* or *tbx6*. *boz* specifies the 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 limiting the anti-neuralizing activity of Bmp morphogens. In addition, by negative regulation of Wnt signaling, *boz* limits posteriorization of neuroectoderm. *bozozok chordino* double mutants exhibit a synergistic loss of head and trunk. This synthetic phenotype is due to dramatically increased Bmp signaling and consequent massive accumulation of cells in the tailbud at the expense of dorso-anterior structures. Therefore, *boz* and *din* act in overlapping pathways that provide the main mechanism to limit Bmp signaling in the zebrafish gastrula and allow for head and trunk development. Notably, Bozozok appears to function by repressing transcription of target genes such as *swr* (*bmp2b*) gene, and as such is the earliest acting repressor that the nascent dorsal axis is using to antagonize ventral influences.

KEY WORDS: *pattern formation, gastrulation, bone morphogenetic protein, Spemann-Mangold organizer.*

Introduction

The Zebrafish Spemann-Mangold Organizer

At the onset of gastrulation the blastoderm of zebrafish embryos takes the shape of an inverted cup covering the animal hemisphere of a large syncytial yolk cell (Kimmel *et al.*, 1995; Solnica-Krezel *et al.*, 1995). Fate mapping experiments revealed that endodermal precursors occupy the most marginal position in the blastoderm, partially intermixed with a broader mesodermal region, and that the endodermal precursors are the first to undergo involution/ingression movements (Warga and Nusslein-Volhard, 1999). Prospective ectodermal cells reside in the animal region partially overlapping with the mesodermal territory (Kimmel *et al.*, 1990). The embryonic shield, a thickening at the dorsal blastoderm margin, has been considered equivalent to the Spemann-Mangold organizer of amphibian embryos (Spemann, 1938), based on its axis inducing potential (Ho, 1992; Oppenheimer, 1936). The embryonic shield is also characterized by expression of a number of genes detected in the dorsal blastopore lip of the frog, or node of amniote embryos (Cho *et al.*, 1991; Glinka *et al.*, 1998; Sasai *et al.*, 1994; Smith *et al.*, 1993). Among those genes are *chordino* (*din*) and *noggin1* (*nog1*), encoding antagonists of ventralizing Bone morphogenetic proteins (Bmp) (Fürthauer *et al.*, 1999; Miller-Bertoglio *et al.*, 1999; Miller-Bertoglio *et al.*, 1997; Piccolo *et al.*, 1996; Schulte-Merker *et al.*, 1997; Zimmerman *et al.*, 1996), and *dickkopf1*

Abbreviations used in this paper: dpf, days post fertilization; hpf, hours post fertilization; YSL, yolk syncytial layer, YSN, yolk syncytial nuclei.

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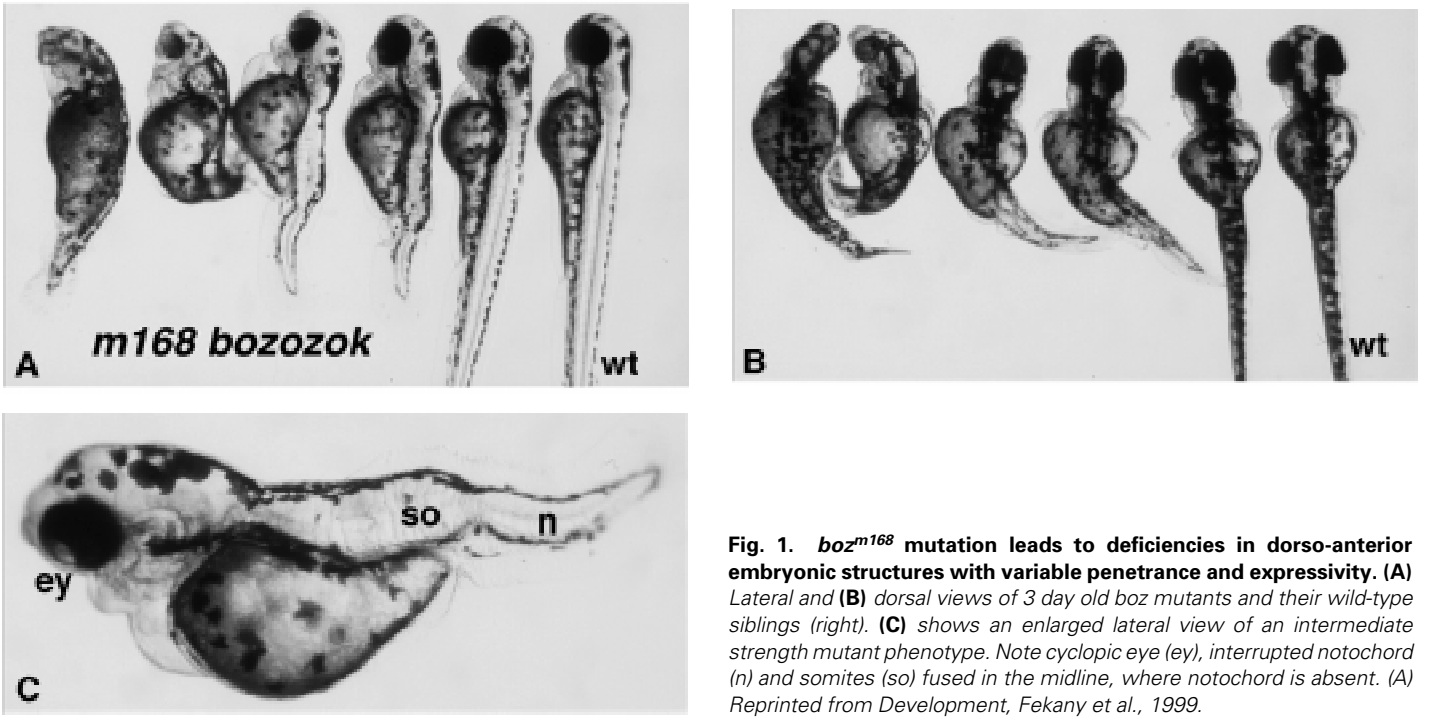


Fig. 1. ***boz*^{m168} mutation leads to deficiencies in dorso-anterior embryonic structures with variable penetrance and expressivity. (A)** *Lateral and* **(B)** *dorsal views of 3 day old boz mutants and their wild-type siblings (right).* **(C)** *shows an enlarged lateral view of an intermediate strength mutant phenotype. Note cyclopic eye (ey), interrupted notochord (n) and somites (so) fused in the midline, where notochord is absent. (A) Reprinted from Development, Fekany et al., 1999.*

(*dkk1*) encoding an antagonists of Wnt signaling (Glinka *et al*., 1998; Hashimoto *et al*., 1999). Furthermore, mutational analyses provided evidence that the function of *din* in the shield region is required for its ability to dorsalize surrounding neuroectoderm and mesoderm (Hammerschmidt *et al*., 1996b). However, transplantations of the embryonic shield to the ventral margin of a host embryo result in formation of partial secondary axes only, in which donor tissue is found predominantly in the notochord, prechordal mesoderm and the floor plate of the neural tube (Ho, 1992; Shih and Fraser, 1996). Recent experiments revealed that the removal of the morphological shield fails to eliminate all cells expressing the organizer-specific genes *goosecoid* (*gsc*) and *floating head* (*flh*) (Cho *et al*., 1991; Schulte-Merker *et al*., 1994a; Stachel *et al*., 1993; Talbot *et al*., 1995), indicating that the physical boundaries of the organizer extend beyond this region (Saude *et al*., 2000). Accordingly, transplantation of a larger piece of the dorsal margin, containing all *gsc* and *flh* expressing cells, leads to formation of complete secondary axes. Surprisingly, the removal of such "enlarged embryonic shield" does not completely block formation of the endogenous axis. The resulting embryos are deficient in axial mesoderm, show significant loss of floor plate and are cyclopic, but their anterior-posterior neural pattern is relatively normal (Saude *et al*., 2000). One interpretation of these results is that activities outside of such a morphologically defined organizer are sufficient to ensure normal AP neural pattern and development of a residual axis. Consistent with this notion, one of the key organizer genes *chordino (din)*, is broadly expressed in the dorsal region of the zebrafish gastrula (Miller-Bertoglio *et al*., 1997; Schulte-Merker *et al*., 1997). Alternatively, some patterning events are completed before the embryonic shield stage, when the extirpation experiments are performed.

In this manuscript we focus on the homeobox gene *bozozok* (*boz*), the function of which is required for the formation of a normal gastrula organizer and subsequent development of dorso-anterior embryonic structures. We review evidence that *boz* promotes organizer formation acting at the blastula stage downstream of β-catenin and synergistically with Nodal signaling. *boz* functions as a transcriptional repressor that directly and/or indirectly limits Bmp and Wnt signaling during organizer formation. In addition, we discuss which organizer activities are compromised in *boz* mutant embryos, and how these deficiencies in specific signaling pathways lead to defined morphological abnormalities.

The homeobox gene *bozozok* is essential for development of dorso-anterior embryonic structures

The mutation *bozozok*^{m168} (Japanese for: *motorcycle thug*) (*boz*) was identified in the course of a large scale mutagenesis screen for zygotic, recessive, lethal mutations that affect early embryonic development in zebrafish (Driever *et al*., 1996; Solnica-Krezel *et al*., 1996). The *boz* phenotype exhibits variable penetrance and expressivity, both decreasing with the age of the female parent. The least affected mutants display notochord discontinuities in the trunk region, while more affected mutants have large gaps in the notochord, associated with normal heads or with synopthalmia (Fig. 1). The most severe mutants lack notochord completely and show a range of head defects from cyclopia and eye reduction, to complete loss of eyes and forebrain, reduction of midbrain and apparent enlargement of the hindbrain region (Fekany *et al*., 1999; Solnica-Krezel *et al*., 1996). A similarly variable phenotype has been reported for an independently isolated *boz*^{i2} mutation that fails to complement *boz*^{m168} (Blagden *et al*., 1997). In addition, the *momo*^{th211} mutation characterized by somewhat milder notochord deficiencies (Hafter *et al*., 1996; Odenthal *et al*., 1996), also falls into the *boz* complementation group (Wilm, T. and LSK, upublished observations).

A homeoprotein Dharma (Japanese for: *a famous buddhist priest*), which had been identified based on its ability to induce ectopic

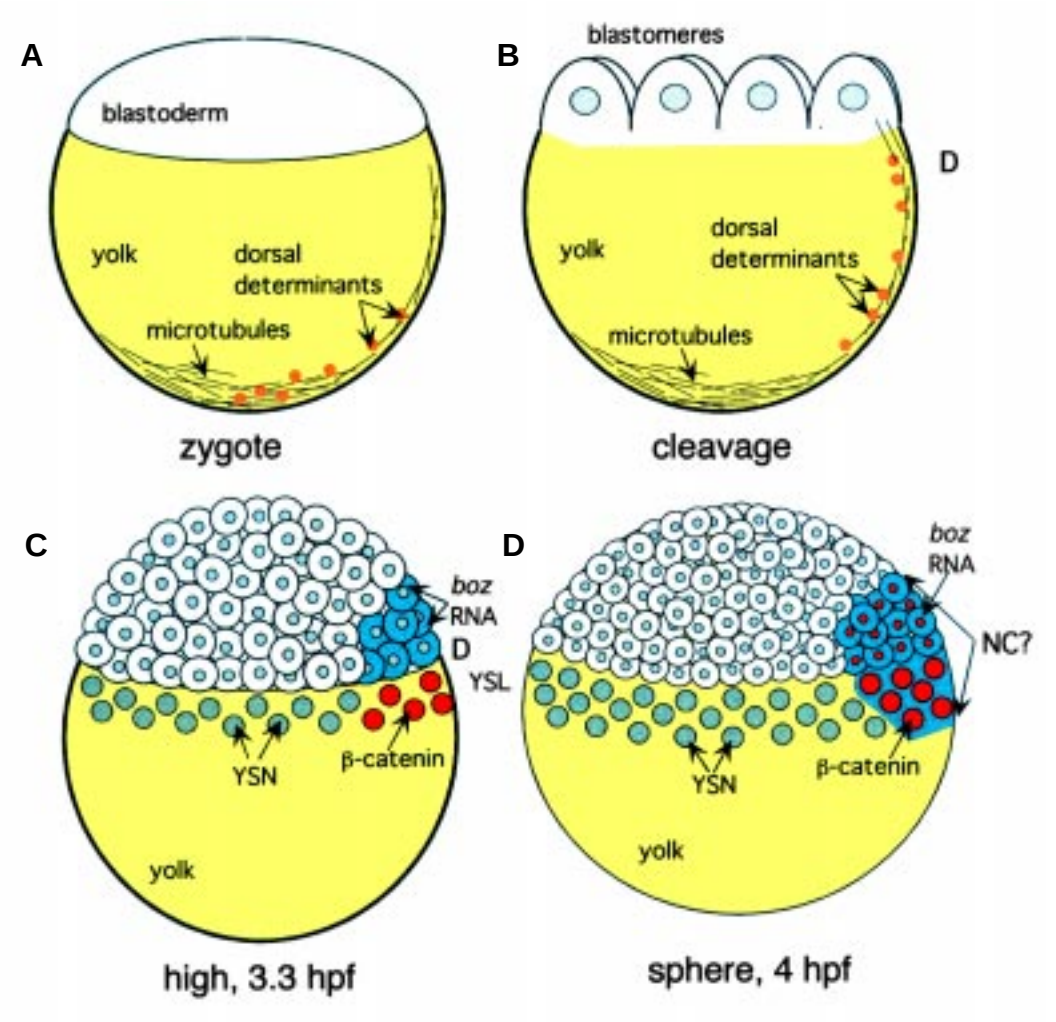
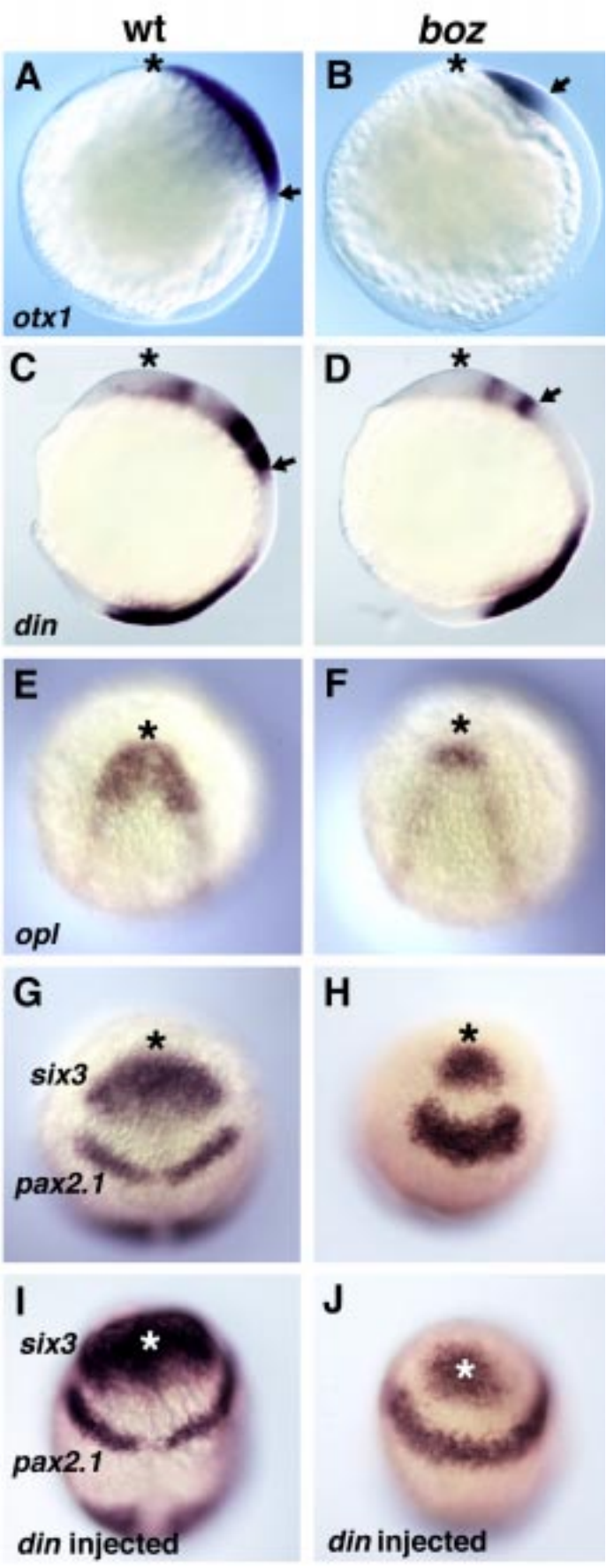


Fig. 2. *boz* acts as a component of the Nieuwkoop center in zebrafish. **(A)** *In the zebrafish zygote, the blastoderm covers a large yolk cell. Dorsal determinants of unknown identity are thought to be located at the vegetal pole of the zygote. Microtubules forming in this region initiate transport of dorsal determinants towards future dorsal blastoderm.* **(B)** *During early cleavage stages, microtubules continue transport of dorsal determinants towards prospective dorsal marginal blastomeres.* **(C)** *At high stage (3.3 hpf), after the onset of zygotic transcription, the zebrafish embryo consists of cellular blastoderm covering large syncytial yolk cell with two rows of syncytial nuclei (YSN). At this stage of development, β-catenin has been detected in the dorsal YSN, whereas boz transcripts are observed in the overlying dorsal blastoderm.* **(D)** *At sphere stage (4 hpf), the yolk syncytial layer (YSL) features three rows of YSN. Nuclear localization of β-catenin and boz transcripts are detected in an overlapping region, encompassing dorsal YSL and dorsal marginal blastomeres. This region most likely corresponds to the Nieuwkoop center (NC) of the zebrafish embryo.*

expression of organizer-specific genes in a non-cell-autonomous manner, emerged as an excellent candidate for being encoded by the *boz* locus (Yamanaka *et al*., 1998). The fact that Dharma is encoded by the *boz* locus is now supported by large body of evidence (Fekany *et al*., 1999). Firstly, *dharma* and *boz* reside in the same interval on Linkage Group 15. Secondly, sequence analysis revealed a nonsense mutation (TGG→TGA transition) within codon 70 of the Dharma ORF, creating a truncated protein that lacks the homeodomain. A restriction fragment length polymorphism created by this mutation has been shown to segregate faithfully with the *boz* phenotype, indicating that *boz* and *dharma* are less than 0.06 cM apart. In addition, the *dharma* sequence is deleted in the γ-irradiation induced boz^{v9} allele. Finally, injections of synthetic *dharma* RNA could completely rescue the *boz* mutant phenotype (Fekany *et al*., 1999). These conclusions have been confirmed by a recent study demonstrating that boz^{m168} is a mutation in an independently isolated *nieuwkoid* gene identical to *dharma* (Koos and Ho, 1998; Koos and Ho, 1999). Sequencing the *boz/dharma/nieuwkoid* ORF from mom^{th211} mutants uncovered a distinct nonsense mutation upstream of the homeodomain (Wilm, T. and LSK, unpublished observations) (Odenthal *et al*., 1996). Together these observations conclusively show that boz^{m168} and mom^{th211} mutations are allelic and disrupt Dharma/Nieuwkoid protein. From that point on we will refer to this locus as *bozozok* (*boz*) and to its protein product as Bozozok.

A truncated amino-terminal portion of the Bozozok protein encoded by the boz^{m168} mutant allele has been shown to be incapable of inducing organizer genes in overexpression experiments, suggesting that this part of the protein is insufficient to mediate the organizer-inducing function of *boz* (Koos and Ho, 1999). Intriguingly, injections of boz^{m168} mutant RNA resulted in a very inefficient induction of ectopic *gsc* expression compared to wild-type RNA. This suggests that a low level of reading through the stop codon during translation of boz^{m168} mutant RNA might produce a full length product (Koos and Ho, 1999), a possibility that should be tested with antibodies to the Bozozok protein. However, the observation that the phenotype of boz^{m168} in trans with the boz^{v9} deletion does not produce a stronger phenotype than that observed for $boz^{m168/m168}$ mutants supports the notion that the boz^{m168} mutation eliminates the majority of the *boz* function (Fekany *et al*., 1999).

Due to the low penetrance of the *boz* phenotype in the progeny of older females, a fraction of homozygous mutants are able to survive to adulthood. Homozygous *boz* mutant females produce 100% wild-type progeny in crosses with wild-type males, indicating that there is no strict maternal requirement for *boz*. However, progeny obtained from $boz^{m168/m168}$ females and heterozygous $boz^{m168/+}$ males usually exhibits a slightly more severe phenotype than the progeny of heterozygous parents, suggesting that mater-



nal *boz* function partially rescues the zygotic mutant phenotype (Fekany-Lee, K. et al., 2000).

Bozozok acts downstream of dorsal determinants and β-catenin

During zebrafish development, the formation of the gastrula organizer and the establishment of dorso-ventral polarity can be traced to the early zygote, in which so-called "dorsal determinants" are thought to reside at the vegetal pole (Fig. 2A)(Mizuno *et al.*, 1999; Mizuno *et al.*, 1997; Ober and Schulte-Merker, 1999). Removal of this region of the zygote results in phenotypes that range from a lack of notochord and eyes, phenocopying the *boz* phenotype, to the complete absence of head and trunk (Mizuno *et al.*, 1999; Mizuno *et al.*, 1997; Ober and Schulte-Merker, 1999). During the first cleavages microtubules forming in the cortical cytoplasmic layer of the yolk transport the dorsal determinants towards the marginal blastomeres contacting the yolk (Fig. 2B). This transport is highly asymmetric and thought to specify the future dorsal side of the embryo. Whereas the molecular identity of the dorsal determinants remains unknown in zebrafish, their translocation is required for nuclear accumulation of β-catenin in the presumptive dorsal side of the embryo (Fig. 2C) (Jesuthasan and Strahle, 1997; Schneider *et al.*, 1996; Trimble and Fluck, 1995).

Several lines of evidence indicate that expression of *boz* is activated via dorsal determinants/β-catenin pathway. During zebrafish development the zygotic transcription is initiated around the 1,000 cell stage (3 hours post fertilization, hpf) (Kane and Kimmel, 1993; Kimmel *et al.*, 1995). Coincident with the midblastula transition, the marginal blastomeres collapse on the yolk cell contributing their cytoplasm and nuclei, thus forming the yolk syncytial layer (YSL). This process creates an embryo, in which a syncytial yolk cell supports the overlying mound of blastomeres (Kimmel and Law, 1985). At high stage (3.3 hpf), two rows of syncytial nuclei are present and β-catenin is detected in an island of these syncytial nuclei in the prospective dorsal region of the yolk syncytial layer (YSL) (Schneider *et al.*, 1996). Intriguingly, at this stage of development, *boz* mRNA is detected in the marginal blastomeres, overlying the region of nuclear accumulation of β-catenin in the YSL (Fig. 2C). After the next division of syncytial nuclei (sphere stage), *boz* expression and nuclear localization of β-catenin are detected in both the dorsal YSL and the marginal blastomeres (Fig. 2C)(Koos and Ho, 1998; Schneider *et al.*, 1996;

Fig. 3. ***boz*** **mutants exibit a reduction of neural anlage, which is a BMP-dependent defect, and excessive posteriorization of neuroectoderm, a BMP-independent defect.** *In the late gastrula, the* orthodenticle1 *(*otx1*) forebrain and midbrain expression domain (Li et al., 1994) is severely reduced in* **(B)** boz *mutants compared to* **(A)** *wild-type siblings. Note that the posterior border of the* otx1 *expression domain is dramatically shifted anteriorly in* boz *mutants. At the onset of segmentation, deficiency of anterior neural fates in boz is further revealed by reduction of* **(C,D)** *the forebrain and midbrain expression domain of* din *(Miller-Bertoglio et al., 1997),* **(E,F)** *the forebrain expression domain of* odd-paired-like (opl) *gene (Grinblat et al., 1998) and* **(G,H)** *the* sine oculis *–related* (six3) *gene (Kobayashi et al., 1998). Posteriorization of neuroectoderm in boz mutants is revealed by anterior shift of hindbrain* din *expression domains (C,D). Overexpression of Chordin results in neuralization of ectoderm in both* **(I)** *wild type and* **(J)**boz *mutants; however it fails to suppress the reduction of forebrain anlage (*six3 *expression domain) (J). (A,B,G,H,I,J) Reprinted from Development, Fekany-Lee et al., 2000.*

Yamanaka *et al*., 1998). Removal of dorsal determinants or interference with their translocation prevents nuclear accumulation of β-catenin as well as expression of the *boz* gene (Ober and Schulte-Merker, 1999). Likewise, overexpression of Axin and Axil, scaffolding proteins that interact with β-catenin and facilitate its degradation, reduce expression of *boz* at blastula and gastrula stages (Shimizu *et al*., 2000). Furthermore, in embryos treated with lithium, an inhibitor of GSK-3β (Hedgepeth *et al*., 1997; Klein and Melton, 1996), there is ectopic nuclear accumulation of β-catenin (Schneider *et al*., 1996), and ectopic expression of *boz* in the YSL (Yamanaka *et al*., 1998). Overexpression of β-catenin results in ectopic activation of *boz* transcription from MBT on, and characterization of the *boz* promoter identified several sites, which are bound efficiently by TCF/Lef1 (Leung et al, 2000). Together, these data demonstrate that *boz* is a direct downstream target of the β-catenin pathway. Accordingly, epistatic analysis demonstrated that activation of the β-catenin pathway in *boz* mutants is unable to suppress the deficiency of axial mesoderm (Fekany *et al*., 1999). *boz* mutant embryos, in which β-catenin has been ectopically expressed, often form secondary axes. These secondary axes however, are deficient in prechordal and chordamesoderm. Similarly, treatments at early blastula stages with lithium lead to massive marginal expression of *gsc*, an organizer-specific gene (Cho *et al*., 1991; Stachel *et al*., 1993), in wild type, but not in *boz* mutant embryos. In addition, *boz* function is not required for the nuclear localization of β-catenin (Fekany *et al*., 1999). These observations are consistent with a model, in which the function of *boz* downstream of β-catenin is essential for organizer formation and subsequent development of axial mesoderm (Fekany *et al*., 1999; Leung *et al*., 2000).

boz acts as a key component of the Nieuwkoop center

The expression pattern of *boz* and the requirement for its function downstream of β-catenin during axis formation establish *boz* as a candidate component of Nieuwkoop center activity in the zebrafish blastula. In amphibian embryos, the Nieuwkoop center was identified based on the ability of dorso-vegetal blastomeres to induce full secondary axes upon transplantation into the ventral hemisphere (Nieuwkoop, 1973). It is thought that the Nieuwkoop center promotes axis formation by inducing, in a non-autonomous manner, the gastrula Spemann-Mangold organizer in the overlying blastomeres (Nieuwkoop, 1973; Wylie *et al*., 1996). However, further transplantation experiments indicated that whereas the greatest axis inducing activity indeed resides in the dorsal vegetal cells, just below the equator of the *Xenopus* blastula, this activity is also broadly distributed throughout the dorsal side of the embryo, including cells that will populate the organizer [reviewed in (Moon and Kimelman, 1998)]. Consistent with this notion, the nuclear localization of β-catenin is also observed in a broad dorsal domain corresponding well with the localization of the dorsal determining activity (Larabell *et al*., 1997; Schneider *et al*., 1996). β-catenin is thought to directly activate the homeobox genes *siamois* and *twin*, which would in turn activate expression of the organizer-specific gene *gsc* by binding to its promoter (Brannon *et al*., 1997; Laurent *et al*., 1997). These studies indicate that the Nieuwkoop center and the Spemann-Mangold organizer might physically overlap. Hence, in the amphibian embryo both non-cell-autonomous and cell-autonomous processes are involved in specification of the Spemann-Mangold organizer by the Nieuwkoop center (reviewed in Moon and Kimelman, 1998).

In the zebrafish embryo the majority of Nieuwkoop center – like activity is thought to reside in the dorsal YSL (reviewed in Driever, 1995; Solnica-Krezel, 1999). Indeed, when blastoderm-free yolk cells are transplanted onto the animal pole of another embryo, they induce ectopic expression of mesodermal markers as well as organizer-specific markers and dictate the dorso-ventral polarity of the host blastoderm (Long, 1983; Mizuno *et al*., 1996; Ober and Schulte-Merker, 1999). Notably, yolk cells from which dorsal determinants were surgically removed at the onset of development retain their mesoderm inducing potential but are incapable of inducing ectopic organizer gene expression upon transplantation (Mizuno *et al*., 1999). Therefore, it is an attractive hypothesis that the Nieuwkoop center activity located in the dorsal YSL induces, via non-autonomous signals, the formation of the Spemann-Mangold organizer in the overlying blastoderm. However, gene expression patterns suggest that in zebrafish, as in the frog embryo, Nieuwkoop center activity might have a broader distribution and that there might be a physical overlap between the Nieuwkoop center and the Spemann-Mangold organizer. As described above, β-catenin accumulates not only in the nuclei of the dorsal YSL but also in the dorsal blastomeres as well (Schneider *et al*., 1996). Furthermore, expression of *boz* and other genes with axis inducing activity like *squint* (*nodal-related 2)* is observed in the YSL and marginal blastomeres (Erter *et al*., 1998; Feldman *et al*., 1998; Rebagliati *et al*., 1998a).

As suggested by its expression pattern, *boz* might act both in dorsal YSL and dorsal blastoderm (reviewed in Kodjabachian and Lemaire, 1998). Expression of *boz* becomes progressively restricted to the YSL by the onset of gastrulation (Koos and Ho, 1998; Yamanaka *et al*., 1998). Moreover, injections of *boz* RNA exclusively into the YSL can induce expression of organizer genes in the overlying blastoderm in wild-type embryos (Yamanaka *et al*., 1998) and can rescue the *boz* mutant phenotype (Fekany *et al*., 1999; Koos and Ho, 1999). Hence, function of *boz* in the YSL is most likely sufficient for normal development of the overlying blastoderm. It is noteworthy, however, that overexpression of *boz* in the entire embryo induces dorsal genes in wild-type and *boz* blastoderm more effectively than RNA injections into the YSL (Fekany *et al*., 1999; Yamanaka *et al*., 1998). In addition, when blastomeres expressing *boz* were transplanted into a blastoderm of another embryo, they induced ectopic organizer gene expression in host cells surrounding the transplant (Koos and Ho, 1998). It follows that *boz* is capable of promoting non-autonomous, organizer-inducing signals in the YSL as well as in blastodermal cells. It will be important to determine the functional significance of *boz* within the blastoderm.

One important physical link between the YSL, the blastoderm and *boz* function might be noninvoluting endocytic marginal (NEM) cells (D'Amico and Cooper, 1997). The NEM cell cluster lies in a superficial position of the dorsal blastoderm margin of zebrafish blastula encompassing enveloping layer cells and one or two layers of underlying deep cells. The close association of NEM cells with dorsal YSL and their endocytic properties are suggestive that these cells might maintain a physical contact with YSL longer than the remaining blastoderm cells (D'Amico and Cooper, 1997). During gastrulation NEM cells move in front of the blastoderm, becoming forerunner cells that lead in epibolic movements towards the vegetal pole. Their descendants generate during segmentation the entire structure of Kupffer's vesicle that forms transiently in the

elongating tail of teleost embryos (Melby *et al.*, 1996). Dorsal forerunner cells and Kupffer's vesicle are not observed in *boz* mutants (Fekany *et al.*, 1999). Since NEM cells form in the region of the embryo expressing the *boz* gene (Koos and Ho, 1998; Yamanaka *et al.*, 1998), it will be important to determine what aspect of NEM/forerunner cells development is dependent on *boz* function.

boz is required for the formation of a complete Spemann-Mangold organizer

In strongly affected *boz* mutants, a morphologically distinct embryonic shield does not form and expression of organizer-related genes is altered (Fekany *et al.*, 1999). Four distinct classes of genes can be distinguished based on how their expression in the organizer region is regulated by *boz*. The first class comprises the majority of known organizer-specific genes, such as *gsc*, *flh*, *lim1*, *axial, noggin1 (nog1)* and others (Fürthauer *et al.*, 1999; Stachel *et al.*, 1993; Strähle *et al.*, 1993; Talbot *et al.*, 1995; Toyama *et al.*, 1995), whose expression is reduced or eliminated in *boz* mutants already at blastula stages, and continues to be deficient throughout gastrulation (Fekany *et al.*, 1999; Koos and Ho, 1999; Sirotkin *et al.*, 2000; Solnica-Krezel *et al.*, 1996). The second class of organizer-specific genes, including *chordino (din)*, show a more severe reduction of expression at the blastula and early gastrula, than at later stages of development (Fekany-Lee, K. et al., 2000). Genes of the third class do not require *boz* for initiation of their expression at the blastula stages but only for the maintenance of their expression in the organizer region during gastrulation. Two *nodal*-related genes, *sqt* and *cyclops* (*cyc*), and the *dickkopf1* (*dkk1)* gene encoding a Wnt antagonist belong to this category (Hashimoto *et al.*, 1999; Sampath *et al.*, 1998; Shimizu *et al.*, 2000; Sirotkin *et al.*, 2000). Finally, *boz* is also required to eliminate dorsal expression of a fourth class of genes, including *wnt8*, *tbx6* and *spadetail* (*spt; tbx16*), which are expressed exclusively in the nonaxial mesoderm of wild-type embryos (Fekany-Lee, K. et al., 2000; Koos and Ho, 1999).

boz cooperates with mesendodermal Nodal inducers as a dorsal modifier during gastrula organizer formation

According to the current model, induction of the *Xenopus* gastrula organizer involves a synergistic action of two maternal components that function as mesendodermal inducer and dorsal modifier (Harland and Gerhart, 1997; Heasman, 1997). In both frog and zebrafish, the maternal β-catenin pathway activated by dorsal deteminants is thought to serve as a dorsal modifier (Moon and Kimelman, 1998). It is tempting to hypothesize that in zebrafish *boz* acts downstream of β-catenin as a key regulator of the zygotic dorsal modifier pathway. On the other hand, *nodal*-related zebrafish genes serve as zygotic mesendoderm inducers [reviewed in (Schier and Shen, 2000)] that cooperate with the pathway regulated by *boz* in organizer formation and function. As discussed above, *boz* function is required for initiation and/or maintenance of expression of many organizer-specific genes. However, *boz* is not essential for induction of mesoderm, as general mesodermal markers are expressed around the blastoderm margin of *boz* mutants at the blastula stages and throughout gastrulation (Fekany *et al.*, 1999; Sampath *et al.*, 1998; Solnica-Krezel *et al.*, 1996). Therefore, *boz* is required for the induced mesoderm to acquire its dorsal-most (axial) character, and to subsequently develop into prechordal mesoderm and notochord (Fekany *et al.*, 1999).

Similarly to *boz*, in mutants deficient in Nodal signaling *sqt*, *cyc*, *sqt;cyc* double mutants and MZ *oep* (embryos mutant for their zygotic and maternal contribution of *one eyed pinnhead*, *oep*, encoding a cofactor for Nodal signal), dorsal-most mesoderm development is compromised or completely blocked (Feldman *et al.*, 1998; Gritsman *et al.*, 1999; Rebagliati *et al.*, 1998b; Sampath *et al.*, 1998; Thisse and Thisse, 1999; also see Schier and Talbot, in this volume, pp. 289-298, for review). However, in contrast to *boz,* mutants deficient in Nodal-signaling lack the dorsal expression of all mesodermal markers tested, including *ntl* and *snail1* (Feldman *et al.*, 1998; Gritsman *et al.*, 1999; Thisse and Thisse, 1999). Therefore, Nodal signaling is required for specification of dorso-lateral mesoderm, whereas *boz* function is essential for this mesoderm to obtain the dorsal most, axial identity. Recent work revealed that the early expression of *sqt* is not altered in *boz* mutants and that injections of *boz/dharma* RNA do not induce ectopic expression of *sqt* in the blastula (Shimizu *et al.*, 2000; Sirotkin *et al.*, 2000). In addition, in Nodal signaling deficient embryos injected with *boz* RNA, *din* expression is upregulated, indicating that *boz* is able to induce directly or indirectly some dorsalizing signals independent of Nodal signaling (Shimizu *et al.*, 2000; Sirotkin *et al.*, 2000). This further suggests that in the early stages of organizer and dorsal mesoderm specification, *nodal*-related genes and *boz* might act in parallel pathways activated by β-catenin (Shimizu *et al.*, 2000). It is noteworthy however, that during gastrulation expression of *cyc* and *sqt* in the embryonic shield region is reduced or missing in *boz* mutants (Sampath *et al.*, 1998; Shimizu *et al.*, 2000; Sirotkin *et al.*, 2000), suggesting a more complex relationship between these genes.

It has been demonstrated that differential levels of Nodal signaling pattern the mesoderm and the organizer, with the highest level of activity required for anterior fates (prechordal mesoderm) and lower activity required for posterior (chordamesoderm) fates (Belo *et al.*, 2000; Gritsman *et al.*, 1999). Since overexpression of Nodal, or an activated TGF-β receptor (TARAM-A) can suppress prechordal and chordamesoderm deficiency in *boz* mutants (Fekany *et al.*, 1999), it is intriguing that *boz* promotes formation of axial mesodermal fates by directly or indirectly ensuring high levels of *sqt* and *cyc* expression in the organizer region.

Bozozok promotes organizer formation as a transcriptional repressor of the *swirl* (*bmp2b*) gene

The various positive and negative effects of *boz* on gene expression during development may be due to direct or indirect effects. In order to understand the role of *boz* during development of dorsal structures in zebrafish, its activities as a transcription factor are of crucial importance. The effects of overexpression of fusion proteins joining the Bozozok homeodomain and a transcriptional activator domain, VP-16, or a transcriptional repressor domain from the *Drosophila engrailed* gene have recently been described (Leung et al., 2000). When ectopically expressed in zebrafish embryos, the VP16-Boz fusion protein can induce *boz* like phenotypes and ventralization. Conversely, the EN-Boz fusion protein can rescue the *boz* mutant phenotype and at higher levels of expression dorsalizes the embryos. The fact that the activator

fusion *VP16-boz* phenocopies the *boz* phenotype, while the repressor fusion resembles native Bozozok protein in its activities, strongly indicates that native Bozozok acts as a transcriptional repressor. These findings are confirmed by tests of *boz* activities in heterologous systems, yeast and *Drosophila*, which demonstrate that *boz* does not act as an activator. Indeed, in a sophisticated assay, it has been shown that *boz* acting as an antimorph (or neomorph) inhibits function of its *Drosophila* homolog *bicoid* and represses transcriptional activation of Bicoid target genes (Leung et al., 2000).

Analysis of the interaction between *boz* and *swirl* (*swr*, *bmp2b) (Kishimoto et al., 1997; Nguyen et al., 1998)* in zebrafish revealed several high affinity binding sites in the *bmp2b* promoter (Leung et al., 2000). Thus the best current model for understanding *boz* action in the blastula is that one of its activities is direct transcriptional repression of *swr* (*bmp2b*). Detailed analysis of changes in expression of *swr* (*bmp2b*)*, bmp4 and bmp7* reveal that only *swr* (*bmp2b*) may be a direct target of *boz* repression. *boz* dependent repression of *swr* (*bmp2b*) is reflected by a small dorsomarginal domain devoid of *swr* (*bmp2b*) transcripts at sphere stage, which is not observed in *boz* mutants (Fekany-Lee et al., 2000; Koos and Ho, 1999). This repressed domain does not appear to be dependent on previous activation of *din* expression (Leung et al., 2000). The direct transcriptional repression of *swr* (*bmp2b*) gene by Bozozok in the zebrafish blastula provides one mechanism by which *boz* limits ventralizing Bmp activity and promotes organizer formation. However, since inhibition of Bmp signaling is unable to suppress the deficiency of axial mesoderm in the strongly affected *boz* mutants, *boz* must advance organizer formation also through a Bmp-independent mechanism (Fekany-Lee *et al.,* 2000).

Inhibition of Wnt signaling by *boz* promotes organizer formation

It has been suggested previously that Wnt signaling during gastrula stages might inhibit dorsal gene expression and organizer formation in frog (Christian and Moon, 1993; Itoh and Sokol, 1999) as well as in zebrafish embryos (Pelegri and Maischein, 1998). Furthermore, inhibition of Wnt signaling by overexpressing dominant negative *Xenopus* Wnt 8 (dnXWnt8) (Hoppler *et al.*, 1996) suppresses deficiencies in *din* and *gsc* expression in the organizer of *boz* mutants (Fekany-Lee *et al.*, 2000). Hence, inhibition of Wnt expression and/or activity in the blastula provides an additional mechanism by which *boz* promotes organizer formation.

boz is required for the ability of the gastrula organizer to inhibit Bmp and Wnt signaling

boz mutants develop an incomplete gastrula organizer and subsequently form an axis with multiple deficiencies. Therefore, linking molecular defects in the rudimentary *boz* organizer with their morphological consequences should enhance our understanding of the mechanisms by which the organizer patterns the germ layers. However, additional roles for *boz* independent of the organizer cannot be excluded at the moment. One of the key functions of the Spemann-Mangold organizer is to inhibit the activity of the ventralizing BMP morphogens through production of secreted proteins, Noggin, Chordin, Cerberus and Follistatin that bind to BMP ligands and interfere with their ability to activate the signal transduction pathway (Bouwmeester *et al.*, 1996; Hemmati-

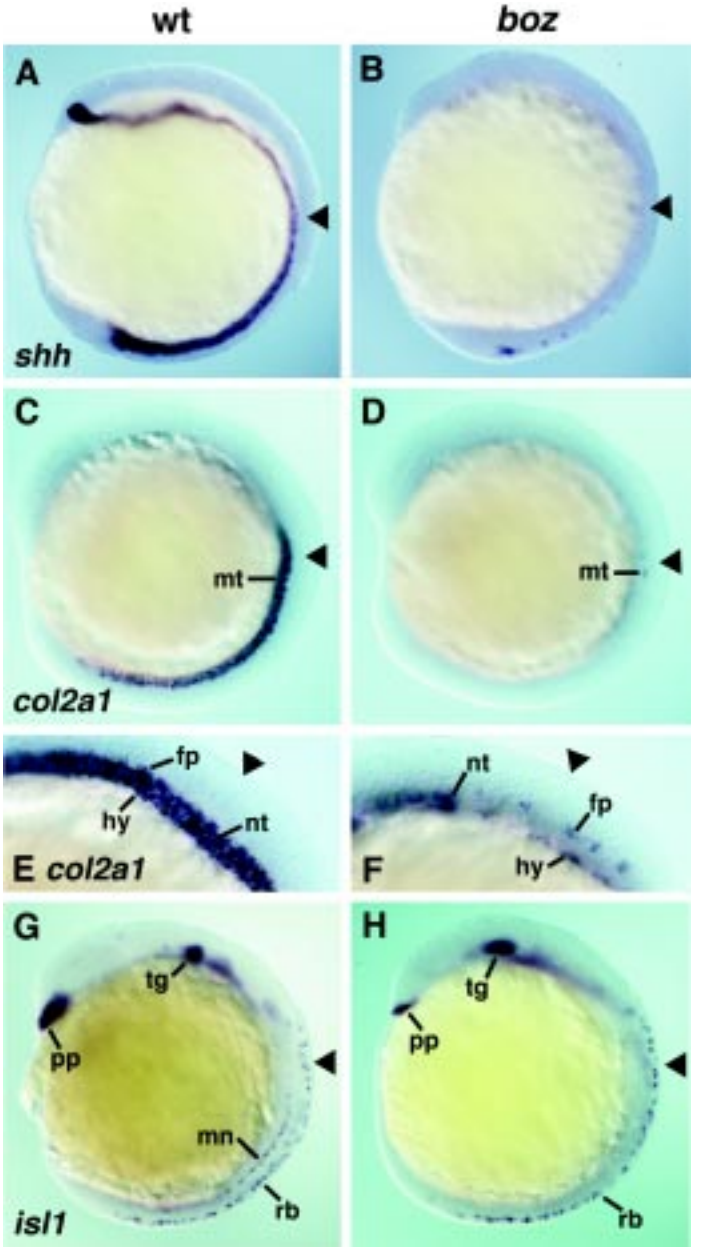


Fig. 4. Development of midline tissues is dependent on *boz* function. *Expression of sonic hedgehog (shh) in developing notochord, prechordal mesoderm and ventral neuroectoderm (Krauss et al., 1993) is dramatically reduced in* **(B)** *strongly affected boz mutants, compared to* **(A)** *wild-type siblings.* **(D)** *A similar effect of boz mutation is observed on the expression of type II collagen gene (col2a1) in midline tissues (mt: notochord, hypochord and floor plate (Yan et al., 1995)) of trunk and tail* **(C,E)**. **(F)** *In moderately affected boz mutants, expression of col2a1 reveals reduced numbers of notochord (nt), hypochord (hy) and floor plate (fp) cells, whose positions along the axis do not coincide with one another.*

Brivanlou and Melton, 1997; Lamb *et al.*, 1993; Piccolo *et al.*, 1999; Sasai *et al.*, 1995; Sasai *et al.*, 1994; Smith *et al.*, 1993). *boz* has been shown to be required for expression of the zebrafish homolog of *chordin*, *chordino* (*din*) at the blastula and early gastrula stages (Koos and Ho, 1999; Shimizu *et al.*, 2000; Fekany-Lee, *et al.*, 2000), as well as for the expression of the zebrafish homolog of *noggin*, *nog1*, in the organizer region (Sirotkin *et al.*, 2000).

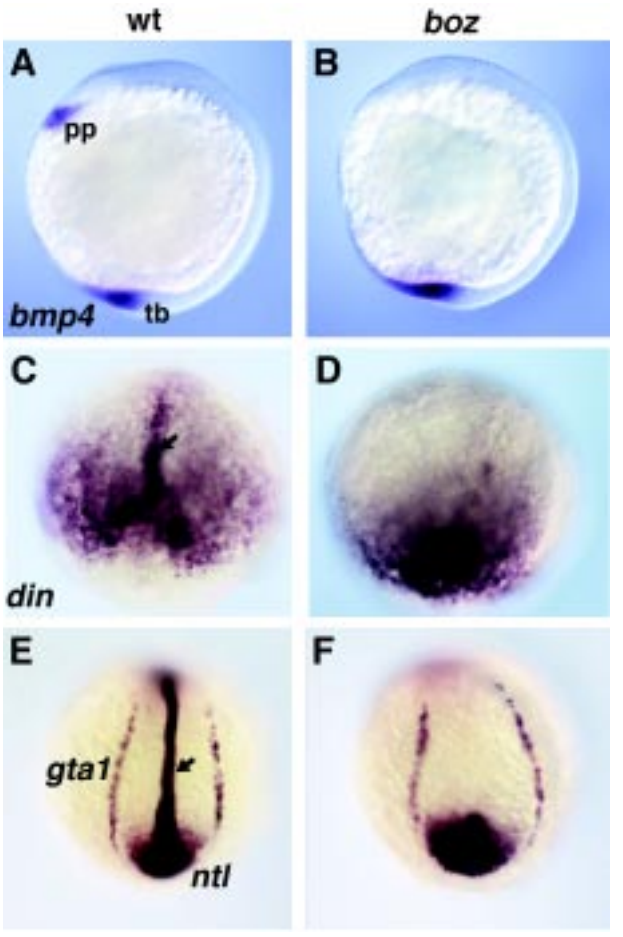


Fig. 5. Mesoderm of *boz* mutants is not strongly ventralized. (A,B) *At the end of gastrulation, expression of the bmp4 gene in the ventro-posterior region of the embryo is not increased in* **(B)** *boz mutants (compared to earlier stages of development), but the prechordal plate (pp) expression domain is absent.* **(C,D)** *Expression of din is not observed in the dorsal midline of boz mutants due to the absence of axial mesoderm. However, the broad lateral expression domain is not significantly reduced in* **(D)** *boz mutants in the late gastrula, in contrast to earlier stages of development.* **(E)** *During early segmentation, the axial mesoderm expression domain of the no tail (ntl) gene (Schulte-Merker et al., 1994b) is not observed in* **(F)** *boz mutants. However expression of the gata1 (gta1) gene in blood precursors (Dietrich et al., 1995) is not significantly increased.*

Consistent with the reduced expression of Bmp antagonists in the organizer region of *boz* mutants, during gastrulation the ventro-lateral expression domains of *bmp2b* and *bmp4* genes are enlarged in *boz* mutants with respect to wild-type siblings. Therefore, *boz* function is required for the ability of the gastrula organizer to inhibit Bmp.

Studies in amphibia revealed that another mechanism the organizer utilizes to pattern germ layers is the inhibition of Wnt signaling (reviewed in Moon *et al.*, 1997). The organizer accomplishes this by production of several secreted factors that are capable of binding to Wnt ligands and preventing their interactions with Frizzled receptors, or antagonizing Wnt signaling by an unknown mechanism. Among those factors are Frzb, bearing similarity to a putative Wnt binding domain of the Frizzled receptors (Leyns *et al.*, 1997; Moon *et al.*, 1997; Wang *et al.*, 1997); Dickkopf-1 (Glinka *et al.*, 1998) and a multifunctional protein Cerberus (Bouwmeester *et al.*, 1996; Piccolo *et al.*, 1999). As discussed previously inhibition of Wnt signaling by *boz* might be important during organizer formation. Furthermore, several observations indicate that the residual organizer that forms in *boz* mutants is defective in its ability to antagonize Wnt signaling. Of the several Wnt antagonists known in *Xenopus*, a homolog of only one of them, *dickkopf1* (*dkk1*) has been reported so far in zebrafish (Hashimoto *et al.*, 1999). *boz* mutants have normal expression of *dkk1* in the dorsal blastoderm margin and YSL at blastula stages, however they exhibit very reduced expression of this gene in the gastrula organizer region (Hashimoto *et al.*, 1999). Moreover, *boz* also antagonizes Wnt signaling by negatively regulating expression of the *wnt8* gene. The *wnt8* gene in zebrafish is expressed in the entire blastoderm margin during early gastrulation, and subsequently becomes excluded from the dorsal midline at midgastrulation (Kelly *et al.*, 1995). This downregulation of *wnt8* expression is not observed in the dorsal margin of *boz* mutant gastrulae, which also appears to exhibit elevated levels of *wnt8* RNA in the lateral blastoderm margin (Fekany-Lee *et al.*, 2000). The overall increase in *wnt8* expression observed in *boz* mutants is intriguing, considering dorsal localization of *boz* RNA (Koos and Ho, 1998; Yamanaka *et al.*, 1998), and might reveal a requirement for *boz* function throughout the embryo. In addition, injection of *boz* RNA at 1-8 cell stage of development results in the reduction or complete elimination of *wnt8* expression in the gastrula, indicating that *boz* function is essential and sufficient for negative regulation of *wnt8* expression (Fekany-Lee *et al.*, 2000). It will be crucial to determine whether the negative regulation of *wnt8* expression is due to a direct repressor activity of the Bozozok protein, or only an indirect, downstream consequence of its action (Fekany-Lee *et al.*, 2000). Together, these studies indicate that *boz* negatively regulates Wnt signaling both by inhibiting expression of the *wnt8* gene and by promoting dorsal expression of Wnt antagonist(s). Effectively, the residual organizer that forms in *boz* mutants is compromised in its ability to inhibit Bmp and Wnt signaling.

Bozozok specifies anterior neural fates by promoting neural induction and negatively regulating posteriorization of neuroectoderm

An intriguing aspect of the *boz* phenotype is the anterior-posterior (AP) neural patterning defect; *boz* mutants are characterized by deficiencies of the anterior-most neural structures, including the telencephalon and apparent expansion of the hindbrain (Fekany *et al.*, 1999). Our recent work revealed that this AP neural patterning defect in *boz* mutants is due to a combination of reduced neural induction and excessive posteriorization of neuroectoderm during gastrulation (Fekany-Lee *et al.*, 2000). The neural induction defect in *boz* is dynamic and is more severe during than at the end of gastrulation. Effectively, the neuroectoderm forming in the late *boz* mutant gastrulae is reduced medio-laterally and is slightly shorter along the AP axis compared to wild-type embryos (Fig. 3 A-F). The reduction of neuroectoderm in *boz* mutants is most likely due to an increase in BMP2b/4 activity and expression. Accordingly, overexpression of Chordin or Noggin can suppress the neural induction defect and completely neuralize *boz* mutant ectoderm (Fig. 3 G-H)(Fekany-Lee *et al.*, 2000).

Notably, the expression of anterior neural markers induced by BMP inhibition in early *boz* gastrulae is not maintained in the neuralized ectoderm of *boz* mutants during late gastrulation (Fig. 3 G-H). Hence, *boz* influences AP neural patterning independent of neural induction. Several observations indicate that a BMP-independent posteriorization of neuroectoderm contributes to the loss of anterior structures in *boz*. At early segmentation stages in *boz* mutants, the MHB and hindbrain anlagen are shifted toward the anterior border of the neuroectoderm (Fig. 3 C,D). Furthermore, fate mapping studies indicate that prospective forebrain cells located at the animal pole of the *boz* gastrula instead contribute to nonneural ectoderm or to the more posterior neural fates (Fekany-Lee *et al.*, 2000).

It has been reported that inhibition of Wnt signaling via overexpression of dominant negative *Xenopus* Wnt8 (dnXWnt8) (Hoppler *et al.*, 1996), or via overexpression of a Wnt-antagonist, Dkk1, rescues forebrain and axial mesoderm deficiencies in *boz* mutants (Hashimoto *et al.*, 1999; Fekany-Lee *et al.*, 2000). Two possibilities present themselves as mechanisms for suppression of AP neural patterning defects by antagonists of Wnt signaling. One possibility is that inhibition of Wnt signaling simply rescues an organizer activity (see above), which in turn suppresses the excessive posteriorization of neuroectoderm by a Wnt- and BMP-independent mechanism.

The second possibility is that continued inhibition of Wnt signaling during gastrulation rescues forebrain development in *boz* embryos injected with *dnXwnt8* RNA. This model deserves consideration for several reasons. Firstly, in zebrafish gastrula, the lateral but not dorsal blastoderm margin exerts a posteriorizing influence upon transplantation into the prospective forebrain region (Woo and Fraser, 1997). Secondly, 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 at midgastrulation (Kelly *et al.*, 1995; Woo and Fraser, 1997). Thirdly, as discussed above, the organizer region in *boz* mutants is deficient in expression of the Wnt antagonist Dkk1 and exhibits ectopic expression of *wnt8* gene (Fekany-Lee *et al.*, 2000; Hashimoto *et al.*, 1999). Finally, there is an ample evidence from amphibia and other systems that Wnt signaling can posteriorize neuroectoderm [reviewed in (Sasai and De Robertis, 1997)], that inhibition of Wnt signaling is important for head development (Glinka *et al.*, 1998; Glinka *et al.*, 1997), and that in *Xenopus engrailed-2* gene is directly regulated by the Wnt signaling pathway (McGrew *et al.*, 1999). Therefore, it is tempting to 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 sets the posterior boundary of anterior neuroectoderm. Subsequently, (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. Hence, Bozozok could play a key role in both steps of the two step AP neural patterning model of Nieuwkoop (Nieuwkoop *et al.*, 1952). *boz* promotes the initial induction of anterior neuroectoderm by limiting the antineural function of the BMP morphogen. Subsequently, *boz* negatively regulates factors such as Wnt8 to promote organizer formation and to limit posteriorization of neuroectoderm (Fekany-Lee *et al.*, 2000).

DV neural patterning defects in *boz* mutants

The reduced neuroectoderm of *boz* mutants exhibits severe dorso-ventral patterning defects that remain only poorly understood. The ventral-most cell types including floor plate (Fekany *et al.*, 1999) and primary as well secondary motorneurons do not form in *boz* mutants (Fig. 4). The deficiency of ventral neural structures is also evident by midline fusion of the bilateral *pax2* expression domains in the prospective midbrain-hindbrain boundary of *boz* mutants (Fig. 3 G,H)(Fekany-Lee *et al.*, 2000)

Can *boz* teach us anything about the mechanisms involved in specification of midline cell types? It is intriguing that in *boz* mutants all the midline structures, notochord, prechordal plate, hypochord and floor plate are reduced or missing completely (Fig. 4 C,D). These deficiencies are correlated with reduction/loss of expression of genes shown in zebrafish and other organisms to be required for ventral neural development. Indeed, expression all of zebrafish *hedgehog* homologs, *shh* (Fig. 4 A,B), *twhh* and *ehh* is reduced or lost in *boz* mutants from the early stages of gastrulation (Currie and Ingham, 1996; Ekker *et al.*, 1995; Fekany *et al.*, 1999; Krauss *et al.*, 1993). Similarly, as discussed above, the expression of the *nodal*-related genes *cyc* and *sqt* in the embryonic shield is also dependent on normal *boz* activity (Sampath *et al.*, 1998; Shimizu *et al.*, 2000; Sirotkin *et al.*, 2000). The less affected mutants show only reduced expression of these genes during gastrulation and subsequently they form a fragmented notochord, hypochord and floor plate along their body axis. It is noteworthy that the fragments of nascent notochord, floor plate and hypochord in such mutants are out of register with one another (Fig. 4F). This is consistent with other studies in zebrafish suggesting that specification of floor plate in zebrafish might occur at earlier stages of gastrulation rather than via vertical signaling from differentiating notochord (Le Douarin and Halpern, 2000; Rebagliati *et al.*, 1998b; Sampath *et al.*, 1998). One attractive scenario is that notochord, prechordal plate, hypochord and floor plate, form an equivalence group, specification of which, is dependent on *boz* activity. Further subdivision of cell fates within this equivalence group might occur via the influence of other signaling systems such as Delta/Notch (Appel *et al.*, 1999), Nodal (Rebagliati *et al.*, 1998b; Sampath *et al.*, 1998) and Hedgehog (Currie and Ingham, 1996; Ekker *et al.*, 1995; Krauss *et al.*, 1993).

Surprisingly normal non-axial mesoderm patterning in *boz*

In striking contrast to the ventralized mutants like *din* or *ogon* (*ogo*) that show expanded mesodermal expression domains of *gta1/2* genes and subsequently develop excess ventral mesodermal fates like blood (Detrich *et al.*, 1995; Hammerschmidt *et al.*, 1996a; Miller-Bertoglio *et al.*, 1999; Solnica-Krezel *et al.*, 1996), *boz* mutants exhibit relatively normal expression of the *gta1* gene during segmentation and have normal amounts of blood at 1 dpf (Fig. 5 E,F). Similarly, *boz* mutants develop normal fin folds in contrast to multiple finfolds featured on the tails of *din* or *ogo* mutants (reviewed in Solnica-Krezel, 1999). This difference in the

degree of ventralization of posterior and lateral mesoderm is likely due to the fact that *boz* mutants show only a mild reduction of *din* expression and small expansion of *bmp2b/4* expression in the late gastrula and during segmentation, compared to *din* or *ogo* mutants (Fig. 5 A-D)(Fisher *et al.*, 1997; Hammerschmidt *et al.*, 1996b; Miller-Bertoglio *et al.*, 1999; Fekany-Lee *et al.*, 2000). Therefore, at the onset of segmentation when *boz* expression is no longer detected (Koos and Ho, 1998; Yamanaka *et al.*, 1998), other genes are likely to promote *din* and downregulate *bmp* expression and activity in *boz* mutants (Fekany-Lee *et al.*, 2000).

Synergistic functions of *boz* and *din* in development of head and trunk

The persistence of residual *din* expression in the *boz* mutant organizer and its recovery in the later stages of gastrulation could explain why axis formation is not completely blocked in these mutants. This notion is supported by the synergistic phenotype of *boz din* double mutants, which exhibit a complete absence of head and trunk. These double mutants develop a tail-like structure with a few posterior somites and very reduced neural tissue (Gonzalez, *et al.,* 2000). Two key observations indicate that this synergistic phenotype is primarily due to an excess of BMP signaling, rather than other redundant functions of *boz* and *din* loci. First, *boz din* double mutants exhibit a dramatic expansion of *swr* (*bmp2b*) and *bmp4* gene expression, compared to single mutants. Second, *boz din;swirl* triple mutants exhibit a *swirl*-like dorsalized phenotype, but with axial mesoderm missing and a deficiency of anterior neuroectoderm typical of the *boz* mutant phenotype. This triple mutant phenotype is identical to that shown by *boz;swr* double mutants, or by *boz* mutants dorsalized by overexpression of *Xenopus* Noggin or Chordin (Fekany-Lee *et al.*, 2000). The simple explanation of these epistatic relationships is that loss of *din* function in *boz* mutants exacerbates ventralization of germ layers primarily by expansion of *bmp* expression and activity.

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