bozozok and squint act in parallel to specify dorsal mesoderm and anterior neuroectoderm in zebrafish

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SUMMARY

In vertebrate embryos, maternal β-catenin protein activates the expression of zygotic genes that establish the dorsal axial structures. Among the zygotically acting genes with key roles in the specification of dorsal axial structures are the homeobox gene bozozok (boz) and the nodal-related (TGF-β family) gene squint (sqt). Both genes are expressed in the dorsal yolk syncytial layer, a source of dorsal mesoderm inducing signals, and mutational analysis has indicated that boz and sqt are required for dorsal mesoderm development. Here we examine the regulatory interactions among boz, sqt and a second nodal-related gene, cyclops (cyc). Three lines of evidence indicate that boz and sqt act in parallel to specify dorsal mesoderm and anterior neuroectoderm. First, boz requires sqt function to induce high levels of ectopic dorsal mesoderm, consistent with sqt acting either downstream or in parallel to boz. Second, sqt mRNA is expressed in blastula stage boz mutants, indicating that boz is not essential for activation of sqt transcription, and conversely, boz mRNA is expressed in blastula stage sqt mutants. Third, boz; sqt double mutants have a much more severe phenotype than boz and sqt single mutants. Double mutants consistently lack the anterior neural tube and axial mesoderm, and ventral fates are markedly expanded. Expression of chordin and noggin1 is greatly reduced in boz; sqt mutants, indicating that the boz and sqt pathways have overlapping roles in activating secreted BMP antagonists. In striking contrast to boz; sqt double mutants, anterior neural fates are specified in boz; sqt; cyc triple mutants. This indicates that cyc represses anterior neural development, and that boz and sqt counteract this repressive function. Our results support a model in which boz and sqt act in parallel to induce dorsalizing BMP-antagonists and to counteract the repressive function of cyc in neural patterning.

Key words: bozozok, squint, cyclops, Nodal, Zebrafish, Dorsal mesoderm, Neural pattern

INTRODUCTION

A cascade of inductive interactions establishes the dorsoventral axis of the vertebrate embryo (Harland and Gerhart, 1997; Moon and Kimelman, 1998; Nieto, 1999). In amphibians, the induction of dorsal structures is initiated by a dorsovegetal signaling center called the Nieuwkoop center (Harland and Gerhart, 1997). The analogous region in teleosts corresponds to blastomeres at the dorsal margin, and the dorsal yolk syncytial layer (YSL), an extrabryonic structure formed when marginal blastomeres fuse with the yolk at the midblastula stage (Long, 1983; Kimmel et al., 1995; Mizuno et al., 1996; Schier and Talbot, 1998).

The Nieuwkoop center is distinguished by the nuclear localization of maternal β-catenin protein (Schneider et al., 1996; Larabell et al., 1997), a transcriptional effector in the Wnt signaling pathway that is required for the initiation of dorsal development (Heasman et al., 1994; Behrens et al., 1996; Molenaar et al., 1996; Fagotto et al., 1997; Pelegri and Maischen, 1998). In zebrafish, β-catenin specifically accumulates in nuclei of dorsal blastomeres and the dorsal YSL (Schneider et al., 1996), where it activates, directly or indirectly, the expression of several zygotic genes that mediate the development of dorsal axial structures (Wylie et al., 1996). The homeobox gene bozozok (boz; also known as dharma and nieuwkoid) is among the first genes activated by β-catenin (Yamanaka et al., 1998; Koos and Ho, 1998; Fekany et al., 1999). Soon after the onset of zygotic transcription, boz is expressed in blastomeres at the dorsal margin. In the late blastula and early gastrula, boz is expressed in the dorsal YSL. Prechordal plate, notochord, floor plate and forebrain are variably disrupted in boz mutants, indicating that boz functions in the development of dorsal mesoderm and ventral neural tube (Schier et al., 1996; Solnica-Krezel et al., 1996; Fekany et al., 1999).

In addition to the transcriptional regulator Boz, a number of secreted proteins play key roles in the development of dorsal mesoderm. One such protein is encoded by squint (sqt), a
member of the nodal-related subclass of the TGF-β superfamily that is essential for early steps in dorsal mesoderm development (Feldman et al., 1998). sqt is expressed in dorsal marginal blastomeres soon after the midblastula transition, and the expression domain then expands to include the dorsal YSL (Erter et al., 1998; Feldman et al., 1998). Analysis of double mutants has revealed that sqt and a second zebrafish nodal-related gene, cyclops (cyc), have partially redundant functions in mesoderm development (Feldman et al., 1998; Rebagliati et al., 1998a; Sampath et al., 1998; reviewed in Schier and Shen, 2000).

Antagonists of ventralizing bone morphogenetic protein (BMP) signals comprise a second class of secreted factors required for the development of dorsal structures. Members of the BMP subclass of the TGF-β superfamily, including Bmp2, Bmp4 and Bmp7, have potent ventralizing activity in explants and when overexpressed in embryos (Dale et al., 1992; Jones et al., 1992; Hemmati-Brivanlou and Thomsen, 1995; Nishimatsu and Thomsen, 1998). Mutational analysis shows that these factors are required for the formation of ventral cell types (Kishimoto et al., 1997; Nguyen et al., 1998; Dick et al., 2000; Schmid et al., 2000). β-catenin and factors downstream of it induce expression of proteins that oppose the action of ventralizing BMPs, including Chordin (Sasai et al., 1994; Piccolo et al., 1996; Pelegrin and Maischein, 1998). Noggin (Smith and Harland 1992; Zimmerman et al., 1996) and Follistatin (Hemmati-Brivanlou et al., 1994).

The cross-regulatory interactions among the genes required for development of dorsal mesoderm are not well defined. For example, overexpression of boz or sqt within the YSL non-autonomously induces dorsal mesoderm formation within the overlying blastoderm (Yamanaka et al., 1998; Koos and Ho, 1998; Feldman et al., 1998), and overexpression of mouse nodal can rescue dorsal mesoderm development in boz mutants (Fekany et al., 1999). Thus boz may activate sqt to induce dorsal mesoderm. Nevertheless, these results do not exclude the possibility that sqt and boz act in parallel during dorsal mesoderm induction, since it has not been determined whether boz is necessary for sqt expression. Additional complexity arises from the observations that boz and sqt can induce chordin expression in zebrafish embryos and Xenopus explants, respectively (Koos and Ho, 1998; Rebagliati et al., 1998b), suggesting that β-catenin could activate chordin through a pathway involving boz, sqt or both.

In order to elucidate the regulatory relationships among the genes required for the development of dorsal structures, we have analyzed the genetic interactions among boz, sqt and cyc. In contrast to previous models in which boz activates sqt expression, we report that the activation of sqt transcription does not require boz function. Instead, we find that boz and sqt are activated in parallel pathways required for dorsal development. Both genes are required for proper expression of noggin1 while either gene is sufficient for the expression of chordin. Ventral fates are markedly expanded in boz;sqt mutants, consistent with the loss of BMP antagonists in these embryos. Whereas boz and sqt single mutants have variable effects on forebrain and axial mesoderm development, boz;sqt double mutants have a severe truncation of the anterior neural tube and a complete loss of axial mesoderm. In striking contrast to boz;sqt double mutants, anterior neural fates are specified in boz;sqt;cyc triple mutants. This indicates that cyc represses anterior neural development, and that boz and sqt counteract this repressive function. Our results support a model in which boz and sqt act in parallel to induce dorsalizing BMP-antagonists and to counteract the repressive function of cyc in neural patterning.

**MATERIALS AND METHODS**

**Mutant alleles and embryo collection**

The mutations utilized in this study, boz<sup>m168</sup>, sqt<sup>c25</sup> and cyc<sup>m294</sup>, have been described previously (Feldman et al., 1998; Rebagliati et al., 1998a; Sampath et al., 1998; Fekany et al., 1999). Embryos were collected from natural matings, raised at 28°C, and staged by morphology as described (Kimmel et al., 1995). Ages in hours postfertilization (h) were normalized to reflect times for a given morphological stage as reported by Kimmel et al. (1995).

**RNA microinjection**

To obtain sense boz mRNA for microinjection, the pCMV-sport dharma plasmid (Yamanaka et al., 1998) was linearized with HpaI and transcribed with SP6 polymerase using the mMessage mMachne kit (Ambion). sqt mRNA was transcribed from the pcBS2+ squint plasmid (Feldman et al., 1998) using SP6 polymerase after linearization with NotI. cyc mRNA was transcribed from the pcBS2+Cyclops plasmid (Rebagliati et al., 1998a) using T7 polymerase after linearization with NotI. RNA was diluted in 5 mg/ml Phenol Red, 0.2 M KCl prior to microinjection. 0.5 nl of RNA was microinjected into 1-4 cell embryos that had been dechorionated by Pronase treatment or 4-8 cell embryos were injected through the chorion. At the appropriate stage embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) and stored in methanol at −20°C.

**In situ hybridization**

Embryos collected for in situ hybridization were fixed in 4% paraformaldehyde in PBS at 4°C and manually dechorionated. Antisense RNA was synthesized and hybridizations were carried out as described (Thisse et al., 1993). Constructs used to synthesize antisense probes for sqt, chordin, cyc, boz, gsc, axial, hgg1, otx2, pax6.1, pax2.1, emx1 and krox20 have been previously described (Krauss et al., 1991a,b; Otxoby and Jowett, 1993; Strähle et al., 1993; Mori et al., 1994; Thisse et al., 1994; Morita et al., 1995; Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997; Feldman et al., 1998; Sampath et al., 1998; Yamanaka et al., 1998). Antisense noggin1 probe was transcribed with SP6 polymerase from Salt-linearized IMAGE zebrafish EST clone fc23h06, which corresponds to the noggin1 gene (Fürthauer et al., 1999).

**Photography and genotyping**

Live embryos were manually dechorionated, anesthetized in Tricaine and photographed in 2.5-3.5% methylocellulose in embryo medium as described (Kimmel et al., 1995). Following in situ hybridization, embryos were stored in methanol at −20°C. Prior to photography embryos were cleansed in benzyl benzoate:benzyl alcohol (2:1), mounted in Canada balsam:methyl salicylate (40:1) and photographed using a Zeiss Axioplan microscope. For genotyping after photograph, embryos were washed twice in benzyl benzoate: benzyl alcohol (2:1), 2-3 times in 100% methanol and once in PBS with 0.1% Tween-20. Genomic DNA was extracted using the QiaAmp Tissue Kit (Qiagen). Following extraction, genomic DNA was precipitated and resuspended in 50-80 µl H2O (embryos younger than 40% epiboly were resuspended in 50 µl). 5 µl of the genomic DNA was used as a template in each PCR reaction. Primers and conditions for genotyping sqt<sup>c25</sup>, cyc<sup>m294</sup> and boz<sup>m168</sup> have been described (Feldman et al., 1998; Sampath et al., 1998; Fekany et al., 1999).
RESULTS

boz requires Nodal-related signals to induce dorsal mesoderm

Previous studies have shown that boz and sqt are expressed in the YSL and dorsal marginal blastomeres and that both boz and sqt can induce dorsal mesoderm in wild-type embryos (Erter et al., 1998; Feldman et al., 1998; Koos and Ho, 1998; Yamanaka et al., 1998). To begin to understand the relationships between boz, sqt and cyc, we tested whether the activity of boz requires Nodal-related signals (Fig. 1). We injected synthetic boz mRNA into wild-type, cyc, sqt and sqt;cyc embryos and assayed the homeobox gene goosecoid (gsc) as a marker for dorsal mesoderm. Microinjection of 75 pg of boz mRNA strongly induces gsc in wild-type embryos (Fig. 1B; Koos and Ho, 1998; Yamanaka et al., 1998) and cyc mutants (data not shown). In contrast, boz can induce gsc only weakly in sqt mutants (Fig. 1D), and no gsc expression is detectable in sqt;cyc mutants injected with boz mRNA (Fig. 1F). While boz is unable to induce dorsal mesoderm in the absence of Nodal signaling, it can still dorsalize sqt;cyc mutants, as indicated by induction of chordin (chd) (Fig. 1H), which is expressed in axial and paraxial mesoderm and dorsal ectoderm in wild-type zebrafish embryos (Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997). Because boz cannot induce dorsal mesoderm in the absence of Nodal-related signals, we conclude that sqt functions either downstream of boz or that sqt and boz act in parallel pathways required for the development of dorsal mesoderm.

boz and sqt are not required to initiate each other’s expression

As one test to distinguish whether Nodal-related signals act downstream of or parallel to boz, we examined sqt and cyc expression in boz mutants. The early phases of sqt and cyc expression are intact in boz mutants (Fig. 2B,G,K), while later expression is decreased (Fig. 2L). In the reciprocal set of experiments, we examined the expression of boz in sqt and sqt;cyc double mutants. At 40% epiboly (5 h) the expression of boz is intact in both sqt and sqt;cyc double mutants (Fig. 2D,E). Thus early expression of sqt and cyc is independent of boz function, and boz expression does not require the activity of Nodal-related signals. These results support the conclusion that the expression of boz and sqt is initiated in parallel.

Expression of noggin1 and goosecoid, but not chordin, requires boz

boz does not regulate the earliest phases of sqt or cyc expression, suggesting that it controls other genes that induce the development of dorsal cell types. To determine if boz is required for the expression of chd or noggin1 (nog1) (Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997; Furthauer et al., 1999), we analyzed their expression in boz mutants. At 30% epiboly (4.7 h) and shield stage (6 h), expression of chd in boz mutants is comparable to wild type (Fig. 3B,D). In contrast, at 30% epiboly, nog1 mRNA is not detectable in boz mutants (Fig. 3F), and expression is reduced or absent at shield stage (Fig. 3H). Thus nog1 expression is dependent on boz function, but other regulators must activate chordin expression. As in previous reports (Solnica-Krezel et al., 1996; Fekany et al., 1999), expression of gsc is reduced in boz mutants at 30% epiboly (Fig. 3J) and shield stage (Fig. 3L).

boz and Nodal-related signals act in parallel to regulate chordin

While expression of chd does not require boz, overexpression of boz strongly induces chd (Koos and Ho, 1998; Fig. 4B). Similarly, both sqt (Rebagliati et al., 1998b; Fig. 4C) and cyc (Fig. 4D) can induce chd, but expression of chd is not altered in sqt or sqt;cyc mutants at 40% epiboly (Fig. 4F,G). Thus overexpression of boz, sqt or cyc is sufficient to induce chd expression, but analysis of boz mutants and single and double mutants for the nodal-related genes shows that they are not essential for chd expression in the late blastula.

To determine if boz and sqt might act redundantly to activate
chordin, we examined chd expression at 35% epiboly (4.8 h) in boz; sqt double mutants. In contrast to the strong chordin expression in boz and sqt single mutants, chordin is only weakly expressed in boz; sqt double mutants (Fig. 5D, compare to B,C). boz; sqt;cyc triple mutants also express low levels of chd RNA (Fig. 5E). These results indicate that boz and sqt act in parallel pathways that independently activate chordin expression.

In contrast to chd, nog1 mRNA levels are reduced in both boz and sqt single mutants (Fig. 5G,H), indicating that both genes are required for nog1 expression in the late blastula. In addition, nog1 transcripts were not detected in boz; sqt double and boz; sqt;cyc triple mutants at 30% epiboly (Fig. 5J).

Anterior truncations and lack of dorsal mesoderm in boz; sqt double mutants

The results described above suggested that boz and sqt act in parallel in the development of dorsal axial structures. This model predicts that boz; sqt double mutants have a stronger reduction of dorsoanterior structures than either single mutant. Both boz and sqt single mutants have variable phenotypes at 24 h, ranging from axial mesoderm defects and cyclopia to wild-type appearance and viability (Fig. 6C-F; Solnica-Krezel et al., 1996; Heisenberg and Nüsslein-Volhard, 1997; Fekany et al., 1999). In contrast, we found that boz; sqt double mutants have a fully penetrant phenotype characterized by the absence of axial mesoderm and a severe anterior truncation of the neural tube (Fig. 6L). The absence of axial mesoderm was confirmed by examination of the notochord and prechordal plate markers axial, cyc, gsc and hgg1 (Fig. 7). Ventral fates are expanded at the expense of these dorsal cell types in boz; sqt mutants, as

Fig. 2. Expression of nodal-related genes in boz mutants and boz in sqt and sqt;cyc mutants. Analysis of the expression patterns of sqt and cyc in boz mutants. (A,B) At sphere stage (4 h), sqt expression is comparable in boz mutants (B) and wild-type siblings (A). (C-E) At 40% epiboly (5 h) expression of boz is comparable in wild-type (C), sqt mutants (D) and sqt;cyc mutants (E). (F-M) At 30% epiboly (4.7 h), the expression patterns of sqt and cyc are indistinguishable in boz mutants (G,K) and their wild-type sibling (F,J). At the start of gastrulation (shield stage, 6 h), expression of sqt and cyc is reduced in boz mutants (I,M). Genotypes were determined following photography (see Materials and Methods). Animal pole views are shown. When evident, dorsal is to the right.

Fig. 3. Expression of dorsal markers in boz mutants. Analysis of the expression patterns of chordin (chd), noggin1 (nog1) and gsc in boz mutants. At 30% epiboly (4.7 h) and shield stage (6 h) chd expression is indistinguishable in boz mutants (B,D) and their wild-type siblings (A,C), while nog1 and gsc are both reduced in boz mutants (F,H,J,L) compared to their wild-type siblings (E,G,I,K). Genotypes were determined following photography (see Materials and Methods). Animal pole views are shown, except for G and H, which are lateral views. When evident, dorsal is to the right. Embryo in G is bozm168/+.
indicated by the expanded expression of the ventral marker gata2 at 80% epiboly (8.1 h) (Fig. 5N).

The neural tube is anteriorly truncated in boz; sqt mutants. Morphological observation showed that the eye is absent but that the otic vesicle, located adjacent to hindbrain rhombomere 5, is present in boz; sqt mutants (Fig. 6I, J). To further characterize the truncations of the neural tube, we examined marker gene expression. Markers of neural fates anterior to the mid-hindbrain junction are not expressed at 24 h (Fig. 8D, E). The loss of the anterior neural plate is apparent at the 2-somite stage (10.7 h), when expression of emx1, a marker of prospective telencephalon, is greatly reduced or absent in boz; sqt mutants (Fig. 9G) and otx2, a marker of the prospective forebrain and midbrain, is severely reduced (Fig. 9L). These results indicate that boz and sqt have overlapping functions required for the specification of the anterior neuroectoderm.

cyc represses anterior neural fates

Since the nodal-related genes sqt and cyc have partially overlapping functions in mesendoderm development, we examined boz; cyc and boz; sqt; cyc mutants. boz; cyc double mutants display cyclopia and a reduced or absent notochord (Fig. 6G). This phenotype is fully penetrant, indicating that
loss of cyc enhances the midline defects of boz mutants (Fig. 6E,F).

The amount of neural tissue is reduced in boz; sqt; cyc triple mutants in comparison to the double mutant combinations (Fig. 6K,L). As in sqt; cyc double mutants (Feldman et al., 2000), the trunk spinal cord is lacking in boz; sqt; cyc triple mutants, as indicated by the absence of pax6.1 expressing spinal cord neurons (Fig. 8K) and the proximity of the hindbrain marker krox20 to the tail (Fig. 8L).

Strikingly, anterior neural fates are present in boz; sqt; cyc mutants despite the lack of the most anterior neuroectoderm in boz; sqt siblings. This is evident from examination of the weakest boz; sqt; cyc mutants, which clearly contain an eye with lens (Fig. 6L), while eyes were never observed in boz; sqt mutants (Fig. 6I). Likewise, expression of pax6.1 is greatly reduced or absent in boz; sqt embryos (Fig. 8D) but present in boz; sqt; cyc mutants at 24 h (Fig. 8J). To determine whether the lack of eyes is due to an early failure to specify anterior neural identities or to a later deterioration of the neural plate, we examined the expression of two anterior neural plate markers, emx1 and otx2 in 2-somite-stage embryos (Fig. 9). emx1 is greatly reduced or not detectable in boz; sqt double mutants (Fig. 9G), while boz; sqt; cyc triple mutants characteristically express emx1 at a comparable level to wild type (Fig. 9H). Similarly, otx2 is strongly reduced at the 2-somite-stage in boz; sqt embryos (Fig. 9L), but is present in boz; sqt; cyc mutants (Fig. 9M). A similar repressive effect of the cyc gene on the anterior neural plate is noted upon comparison of sqt single and sqt; cyc double mutant phenotypes: the expression of emx1 and otx2 is expanded in sqt; cyc double mutants compared to sqt single mutants (Fig. 9). In contrast, boz, cyc and boz; cyc mutants have emx1 expression that is similar to wild type.

Fig. 6. Genetic interactions among boz, sqt and cyc. Lateral views of live embryos with the indicated genotypes at 28-30 h. Arrows indicate notochord (A,B,D,F) or notochord rudiment (E). Two examples of genotypes with variable phenotypes are shown. (A) Wild type. (B) cyc mutant. (C,D) sqt mutants have a variable phenotype; some are cyclopic with a reduced or absent notochord (C), but often sqt mutants have a wild-type notochord and well separated eyes (D). (E,F) The boz mutant phenotype is also variable. Some boz mutants are cyclopic with a reduced or absent notochord (E), but often boz mutants have a wild-type notochord and well separated eyes (F). (G) boz;cyc double mutant. (H) sqt;cyc double mutant; arrowhead indicates large cyclopic eye. (IJ) Severe and less severe boz; sqt double mutants. Eyes are never observed but there is some variability in the extent of the neural tube. White arrowheads indicate position of the otic vesicle. (K,L) Severe and less severe boz; sqt; cyc triple mutants. No eye is present in K but a small cyclopic eye is evident in L (arrowhead). Genotypes were determined following photography by PCR (see Materials and Methods), except for cyc and sqt; cyc mutants, which were scored morphologically.

Fig. 7. Dorsal mesodermal derivatives are absent in boz; sqt double mutants. Dorsal (A-D) and lateral (E-H) views are shown. Comparison of expression pattern of markers of dorsal mesodermal derivatives by in situ hybridization in (A,C,E,G) wild-type and (B,D,F,H) boz; sqt double mutant embryos at (A-F) 80% epiboly (8.1 h) or (G,H) bud stage (10 h). Probes and genotypes are indicated. Axial mesoderm expression of axial and cyc was not detected in boz; sqt double mutants (B,D). Note that the punctate staining of the endoderm is comparable in wild-type (A) and boz; sqt double mutants (B). The prechordal plate is absent in boz; sqt double mutants, as revealed by gsc (F) and hgg1 (H) staining. Genotypes were determined following photography (see Materials and Methods). The wild-type embryos in A and E are boz<sup>m168/+; sqt<sup>c35</sup>+, and the embryos in C and G are sqt<sup>c35</sup>+.
DISCUSSION

boz and sqt act in parallel pathways required for the specification of dorsal mesoderm and anterior neural fates

The functions of boz and sqt are required for early events in dorsal mesoderm development in zebrafish (Solnica-Krezel et al., 1996; Heisenberg and Nüsslein-Volhard, 1997; Feldman et al., 1998; Koos and Ho, 1998; Yamanaka et al., 1998; Fekany et al., 1999). As the regulatory interactions between these genes were unclear, we performed a genetic analysis to study their role in dorsal development and neural patterning. Three lines of evidence indicate that boz and sqt act in parallel and have overlapping roles in the development of dorsal mesoderm and anterior neuroectoderm. First, boz requires Nodal-related signals to induce dorsal mesoderm, because the ability of boz overexpression to activate ectopic gsc expression is reduced in sqt and eliminated in sqt;eyc mutants (Fig. 1). Second, sqt expression at the late blastula stage is not reduced in boz mutants (Fig. 2), demonstrating that boz is not an essential early regulator of sqt transcription. Conversely, boz expression in the blastula is not affected in sqt or sqt;eyc mutants (Fig. 2). Third, whereas boz and sqt single mutants have variable phenotypes, and some homozygous boz and sqt single mutants survive to adulthood, boz;sqt double mutants have a strong and fully penetrant loss of axial mesoderm and anterior structures. These results demonstrate that expression of sqt and boz is activated in parallel and exclude models in which sqt functions downstream of boz in a simple linear pathway.

Our analysis suggests that the boz and sqt pathways converge on a common target, the secreted BMP antagonist Chordin. sqt is a potent inducer of chordin expression (Rebagliati et al., 1998b; Fig. 4), but chordin mRNA levels are not reduced in sqt or sqt;eyc mutants at blastula stages. Similarly, boz function is not required for chordin expression in the late blastula (Fig. 3), despite the fact that boz activates chordin expression (Fig. 4; Koos and Ho, 1998). In boz;sqt double mutants, chordin expression is markedly reduced. These results indicate that sqt and boz have redundant roles in maintaining normal chordin expression levels.

chordin is not the only dorsalizing factor that is a target of the boz and sqt pathways. noggin1 expression is greatly reduced in boz and sqt single mutants, and in boz;sqt double and boz;sqt;eyc triple mutants (Fig. 5). These results indicate that both sqt and boz are required for noggin1 expression, whereas chordin expression requires either boz or sqt. Thus it seems that the dorsalizing activities of boz and sqt are mediated, at least in part, by the activation of BMP antagonists. In addition, inhibition of Wnt signals may be an important consequence of boz and sqt action, because expression of dkk1, which encodes a secreted Wnt antagonist with potent head-inducing activity, is reduced in gastrula-stage boz and sqt mutants (Glinka et al., 1998; Niehrs, 1999; Hashimoto et al., 2000). The finding that boz and sqt

Fig. 8. Analysis of neural markers in boz;sqt, sqt;eyc and boz;sqt;eyc mutants. Gene expression was examined by whole-mount in situ hybridization of (A-C) wild-type, (D-F) boz;sqt, (G-I) sqt;eyc and (J-L) boz;sqt;eyc embryos at 24 h. Lateral views are shown. (A,D,G,J) Expression of pax6.1, which is absent or severely reduced in boz;sqt mutants (D), (B,E,H,K) pax2.1 marks the optic stalk, mid-hindbrain border (arrowhead) and hindbrain in wild-type (B) and sqt;eyc embryos (H). The optic stalk is absent in boz;sqt double mutants (E) and the mid-hindbrain boundary appears to be at the most anterior part of the neural tube. In boz;sqt;eyc mutants (K), the optic stalk expression is absent but the neural tube extends anterior to the mid-hindbrain junction. (C,F,L) krox20 marks hindbrain rhombomeres R3 and R5 in wild-type (C), boz;sqt (F), sqt;eyc (I) and boz;sqt;eyc (L) embryos.
activate common downstream genes might explain why injection of mouse nodal mRNA can induce dorsal mesoderm formation in boz mutants (Fekany et al., 1999). Based on this result, it was previously suggested that sqt acts downstream of boz in a linear pathway triggered by β-catenin. However, the phenotype of boz; sqt double mutants does not support this interpretation, because double mutants should resemble the single mutants if boz acted upstream of sqt in a simple linear pathway. We suggest that in the absence of boz, overexpression of nodal activates shared target genes, thus mimicking the combined effects of boz and sqt. This interpretation is consistent with the parallel and partially overlapping role of boz and sqt deduced from our genetic analysis.

It is not clear whether Boz directly activates transcription of chd, nog1, and other dorsally expressed genes, or whether it acts by way of intermediary factors. In support of the latter possibility, Boz shares a short sequence motif with transcriptional repressors (Koos and Ho, 1998), and boz is required to repress bmp2b expression in the dorsal region of the embryo (Koos and Ho, 1999). Thus Boz may directly repress genes with ventralizing activity such as bmp2b, thereby indirectly activating chd and other dorsally expressed genes.

cyclops inhibits anterior neural fates

Seemingly contradictory roles have been proposed for Nodal signals in anterior neural patterning. In the mouse, nodal is required for anterior development (Varlet et al., 1997), whereas evidence from Xenopus suggests that inhibition of Nodal activity is required for the specification of anterior fates (Bouwmeester et al., 1996; Piccolo et al., 1999). Our analysis in zebrafish provides genetic evidence that repression of a Nodal-related signal (cyc) is important to promote anterior patterning. Comparison of the phenotypes of boz; sqt and boz; sqt; cyc mutants demonstrates that the cyc gene represses anterior neural development, and that boz and sqt counteract this repressive function. In particular, anterior neural fates are absent in boz; sqt double mutants, indicating that forebrain development requires the parallel pathways in which these genes act (Figs 8, 9). In contrast, anterior neural fates are specified in boz; sqt; cyc triple mutants (Figs 8, 9), despite the severe reduction of the neural tube in these embryos. Thus the inactivation of cyc compensates for the loss of boz and sqt function in the development of anterior neural fates. These results support and extend a model derived from the study of the putative head inducer Cerberus (Bouwmeester et al., 1996). It has been proposed that there is an early requirement for Nodal in the induction of head organizer genes including cerberus, which later inhibits Nodal, along with BMP and Wnt activity (Piccolo et al., 1999). Since boz and sqt are required for anterior development, we suggest that sqt corresponds to the early Nodal-related signal that participates in the induction of.
of the head organizer. In contrast, the later activity of Cyc represses anterior development, and inhibition of this function allows specification of anterior fates. cyc mRNA is not expressed in boz:sqt mutants at 80% epiboly (Fig. 7D), suggesting that the cyc gene acts to repress anterior development prior to midgastrulation.

If the cyc gene acted to repress the neural inducers chd and nogl, this could explain the restoration in boz and both genes are required for nodal and the mesoderm in sqt posteriorizing signals that results from the diminution of mutants. According to this view, anterior fates are restored in signals accounts for the loss of anterior neural fates in mutants (Fig. 7), and ventral fates are expanded (Fig. 5). An alternative possibility, because these BMP antagonists are not expressed double mutants. However, our results do not support this explanation derives from the finding that nonaxial mesoderm double mutants at the late blastula stage (Fig. 5). Although the BMP antagonists are expressed in double mutants, they allow specification of anterior fates.

In summary, our results suggest that the homeobox gene boz and the nodal-related gene sqt act in a complex regulatory network that controls dorsal development in zebrafish. These genes act in parallel to activate the BMP antagonist Chordin, and both genes are required for noggin expression, boz and sqt also activate pathways that block Nodal signaling, thus allowing the development of anterior neural fates.

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