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The homeobox genes *vox* and *vent* are redundant repressors of dorsal fates in zebrafish

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SUMMARY

Ventralizing transcriptional repressors in the Vox/Vent family have been proposed to be important regulators of dorsoventral patterning in the early embryo. While the zebrafish genes *vox* (*vega1*) and *vent* (*vega2*) both have ventralizing activity in overexpression assays, loss-of-function studies are needed to determine whether these genes have distinct or redundant functions in dorsoventral patterning and to provide critical tests of the proposed regulatory interactions among *vox*, *vent* and other genes that act to establish the dorsoventral axis. We show that *vox* and *vent* are redundant repressors of dorsal fates in zebrafish. Mutants that lack *vox* function have little or no dorsoventral patterning defect, and inactivation of either *vox* or *vent* by injection of antisense morpholino oligonucleotides has little or no effect on the embryo. In contrast, embryos that lack both *vox* and *vent* function have a dorsalized phenotype. Expression of dorsal mesodermal genes, including *chordin*, *goosecoid* and *bozozok*, is strongly expanded in embryos that lack *vox* and *vent* function, indicating that the redundant action of *vox* and *vent* is required to restrict dorsal genes to their appropriate territories. Our genetic analysis indicates that the dorsalizing transcription factor Bozozok promotes dorsal fates indirectly, by antagonizing the expression of *vox* and *vent*. In turn, *vox* and *vent* repress *chordin* expression, restricting its function as an antagonist of ventral fates to the dorsal side of the embryo. Our results support a model in which BMP signaling induces the expression of ventral genes, while *vox* and *vent* act redundantly to prevent the expression of *chordin*, *goosecoid* and other dorsal genes in the lateral and ventral mesendoderm.

Key words: *vox*, *vent*, *bozozok*, *chordin*, *goosecoid*, *bmp*, Gastrulation, Morpholino, Zebrafish

INTRODUCTION

The asymmetric action of β-catenin protein establishes the dorsoventral axis of vertebrate embryos. In zebrafish and *Xenopus*, maternal β-catenin accumulates in dorsal nuclei, where, in concert with the HMG-box protein Tcf/Lef, it activates the expression of zygotic genes that specify dorsal positional identity (reviewed by Moon and Kimelman, 1998; Kodjabachian et al., 1999). Among the first genes activated by β-catenin in zebrafish is the homeobox gene *bozozok* (*boz*), which is expressed in the dorsal blastomeres and dorsal yolk syncytial layer, a structure that can induce and pattern the mesoderm and endoderm in teleost embryos (Yamanaka et al., 1998; Koos and Ho, 1998). Mutational analysis indicates that *boz* is required for the development of dorsal structures, including the axial mesoderm and anterior central nervous system (Fekany et al., 1999).

Ventral fates are induced by members of the bone morphogenetic protein (BMP) family, a subclass of the transforming growth factor β superfamily, including BMP2, BMP4 and BMP7 (for reviews, see Harland and Gerhart, 1997; Dale and Wardle, 1999; Kodjabachian et al., 1999). In *Xenopus*, explant and overexpression assays show that BMPs have ventralizing activity, promoting the formation of epidermis and ventral mesoderm at the expense of neuroectoderm and dorsal mesoderm. The zebrafish mutations *swirl* (*swr*) and *snailhouse* (*snh*) inactivate the *bmp2b* and *bmp7* genes, respectively, and produce strongly dorsalized embryos in which paraxial mesoderm and the neural plate are expanded at the expense of ventral structures (Mullins et al., 1996; Kishimoto et al., 1997; Nguyen et al., 1998; Dick et al., 2000; Schmid et al., 2000). A number of dorsally expressed proteins antagonize the ventralizing action of BMPs, thereby promoting the formation of dorsal fates. One such BMP antagonist is Chordin (Sasai et al., 1994), a secreted factor that binds and inactivates BMPs (Piccolo et al., 1996). Zebrafish *chordin* (*chd*) mutants are ventralized, such that ventral cell types, including blood and epidermis, are expanded, while

more dorsal structures such as neural plate and paraxial mesoderm are reduced (Hammerschmidt et al., 1996a; Schulte-Merker et al., 1997).

The *vox*/*vent*-class homeobox genes have also been implicated in patterning the dorsoventral axis. In *Xenopus*, *Vox*/*Xvent-2/Xom* (referred to here as *Vox*) is expressed ubiquitously in the late blastula, and soon thereafter transcripts are excluded from dorsal territories including the dorsal mesoderm and presumptive neural plate (Ladher et al., 1996; Onichtchouk et al., 1996; Papalopulu and Kinter, 1996; Schmidt et al., 1996). Transcripts of *Xvent-1* (and the closely related gene *PV.1*) are also excluded from dorsal territories, but, in contrast to *Vox*, expression is largely confined to the margin (Gawantka et al., 1995; Ault et al., 1996). Overexpression of either Vox or Xvent-1 can ventralize the embryo, mimicking the effects of BMP overexpression, but differences in the extent of ventralization suggest that there may be quantitative or qualitative differences in the activities of the two genes (Gawantka et al., 1995; Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996). Analysis of fusion proteins with transcriptional activator and repressor domains shows that Vox and Xvent-1 can act as transcriptional repressors (Onichtchouk et al., 1998; Melby et al., 1999; Trindade et al., 1999), although one study has suggested that Vox directly activates transcription of the *Xvent-1* and *BMP4* genes (Schuler-Metz et al., 2000). Vox and Xvent-1 were proposed to be key transcriptional mediators of ventralizing BMP signals, because BMP4 induces expression of both genes, and overexpression of either gene can counteract the dorsalizing effects of a dominant negative BMP receptor (Gawantka et al., 1995; Ault et al., 1996; Ladher et al., 1996; Onichtchouk et al., 1996; Schmidt et al., 1996; Onichtchouk et al., 1998). There is also evidence, however, that Vox and Xvent-1 act upstream of BMP4, supporting the possibility that BMP4 and Vox/Vent transcription factors may promote each other's expression in a positive feedback loop (Onichtchouk et al., 1996; Schmidt et al., 1996; Onichtchouk et al., 1998). Despite the evidence that Vox and Xvent-1 have similar activities as transcriptional repressors in overexpression assays, previous work has not established whether these genes have distinct or redundant functions.

Overexpression studies have also implicated two zebrafish *vox*/*vent*-class homeobox genes, known as *vox* (or *vega1*) and *vent* (or *vega2*), in dorsoventral patterning (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). The homeodomains of the Vox and Vent proteins are about 70% identical to each other and to *Xenopus* Vox and Xvent-1. Based on overall sequence identity, it is difficult to determine which zebrafish and frog genes are orthologous, but similarities in expression pattern suggest that zebrafish *vox* corresponds to *Vox* and that zebrafish *vent* corresponds to *Xvent-1* (Melby et al., 2000). The *vox* gene is widely expressed soon after the midblastula transition, and transcripts are not detectable in dorsal cells by the late blastula stage (Kawahara et al., 2000a; Melby et al., 2000). Expression of *vent* is first detected at the late blastula stage in the ventrolateral marginal region (Kawahara et al., 2000b; Melby et al., 2000). BMP signals are required for the maintenance of *vox* and *vent* expression in the late gastrula, and the Boz homeodomain protein acts in the late blastula to establish the asymmetric distribution of *vox* and *vent* by inhibiting their expression in dorsal marginal cells. As with the *Xenopus* genes, overexpression of *vox* or *vent* can ventralize the embryo, and analysis of activator and repressor domain fusion proteins suggests that Vox and Vent can act as transcriptional repressors (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). Possible downstream genes include dorsal genes such as *dkk1*, *squint*, *cyclops*, *floating head* (*flh*), *chd* and *goosecoid* (*gsc*) (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000), but it is not clear which of these are crucial targets for Vox or Vent. Although *vox* and *vent* may control similar or identical target genes, differences in the activities of *vox* and *vent* in overexpression assays suggest that these proteins may have different regulatory functions (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). Loss-of-function studies are required to address what aspects of dorsoventral patterning require the functions of *vox* and *vent*, to determine the extent to which these functions overlap, and to provide crucial tests of the proposed regulatory interactions among the *vox*, *vent*, *boz*, *chd* and *bmp* genes.

We report that *vox* and *vent* are redundant repressors of dorsal fates in zebrafish. In a genetic screen for mutants with altered dorsoventral patterning, we recovered two deletions of LG13, which our mapping experiments show is the location of both *vox* and *vent*. In some genetic backgrounds, homozygous deletion mutants are strongly dorsalized, and the expression of dorsal marker genes expands to include lateral and ventral blastomeres. In a second screen, we recovered a point mutation, vox^{st9}, that inactivates *vox*. Most homozygous vox^{st9} individuals have a wild-type phenotype, but a few are dorsalized to a variable degree. Transheterozygotes for the point mutation and deletion are also variably dorsalized, but the fraction of mutants displaying a dorsalized phenotype is higher than for homozygous vox^{st9} mutants. Whereas most *vox* mutants are wild type in appearance, injection of an antisense morpholino oligonucleotide (MO) that reduces *vent* function, strongly dorsalizes *vox* mutants. Similarly, wild-type embryos injected with MO for both *vox* and *vent* are strongly dorsalized, while injection of either MO alone has little or no effect. We show that the redundant action of *vox* and *vent* is required to restrict dorsal mesodermal genes to their appropriate territories. Using both mutants and MO injection, we have analyzed the interactions between *vox*, *vent*, *boz* and *chd*. Our results demonstrate that a key function of *boz* is to repress the expression of *vox* and *vent*, suggesting that *boz* promotes dorsal fates indirectly, by inhibiting the ventralizing action of *vox* and *vent*. The redundant action of *vox* and *vent* antagonizes *chordin* expression, and the expanded action of *chd* is a key reason that ventral gene expression is reduced in embryos that lack *vox* and *vent* function. Our results provide genetic evidence that *vox* and *vent* function in parallel with *bmp2b* signaling to establish ventral fates in the late blastula. BMP signaling may act to induce the expression of ventral gene expression, while *vox* and *vent* act redundantly to prevent the expression of *chordin*, *goosecoid* and other dorsal genes in the lateral and ventral mesendoderm.

MATERIALS AND METHODS

Mutagenesis and screening

To isolate new mutations that disrupt dorsoventral patterning in

zebrafish, we sought mutants among the F_2 haploid offspring of F_1 fish generated by postmeiotic mutagenesis with the chemical mutagen ENU as described (Imai et al., 2000). To detect mutants with dorsoventral patterning defects, we analyzed haploid embryos by in situ hybridization with a probe for *gsc* (51 clutches), by morphological inspection (23 clutches), or both (187 clutches). Four mutations recovered from this screen have been described previously (Dick et al., 2000; Imai et al., 2000). We now report two new mutations, *T(LG13)*^{st7} and *T(LG13)*^{st8}, that strongly expand *gsc* expression and produce a dorsalized phenotype. In the original F_2 haploid clutches screened, these mutations caused expanded *gsc* expression in a small fraction of the embryos (5/62 for *st7*, 5/73 for *st8*). In subsequent generations, both mutations produced dorsalized phenotypes in non-Mendelian ratios in haploid embryos that are characteristic of translocations (*st7*: 24% dorsalized, *n*=715; *st8*: 15%, *n*=399). Mapping studies (see below and data not shown) indicate that both mutations are translocations involving LG13, consistent with previous evidence that postmeiotic mutagenesis with ENU can produce chromosomal rearrangements in addition to point mutations (Riley and Grunwald 1995; Imai et al., 2000). For both mutations we obtained unbalanced translocation heterozygotes that transmit deletions as Mendelian recessive lethal mutations (*st7*: 50% dorsalized in haploid embryos, *n*=324; *st8*: 50%, *n*=70; see Table 1 for segregation data in diploids). We refer to these deletions as *Df(LG13)*^{st7} and *Df(LG13)*^{st8}, or, for simplicity, *Df*^{st7} and *Df*^{st8}.

We conducted a second genetic screen to identify point mutations in genes located within the region deleted by *Df*^{st7}. In this screen, heterozygous *Df*^{st7}/+ male tester fish were crossed to female F_1 fish generated by premeiotic ENU treatment, which induces almost exclusively point mutations. To mutagenize spermatogonial cells, wild-type males (TL, Haffter et al., 1996) were treated with 3 mM ENU three times, according to the standard protocol (van Eeden et al., 1999). The F_2 progeny were screened at approximately 24 hours for dorsalized phenotypes by morphological criteria. The use of male *Df*^{st7}/+ tester fish avoided the weak dominant maternal effect of *Df*^{st7} (Table 1).

Genetic mapping and analysis of mutant alleles

Genetic mapping and analysis of translocations and deletions were done as described (Talbot and Schier, 1999; Imai et al., 2000; Kelly et al., 2000). Primers for simple sequence length polymorphism (SSLP) markers were obtained from Research Genetics (Huntsville, AL). Polymorphisms in *vox* and *vent* were scored in the HS meiotic mapping panel as described (Kelly et al., 2000; http://zebrafish.stanford.edu); the primers used for *vox* were 5′-GATCAGGCCTATAAGCTCG-3′ and 5′-GCAGTTTCTGATCTGTATCTGTG-3′; and for *vent* the primers were 5′-GAAACTCAGGTGAAGACGTGG-3′ and 5′-CCTTTTGTGCATAGAATTATCGCAC-3′. The *vox*^{st9} lesion was identified by direct sequencing of RT-PCR products as described (Talbot and Schier, 1999). To confirm the mutation, a *vox* fragment was amplified from genomic DNA of wild-type and dorsalized mutant (*Df*^{st7}/*vox*^{st9}) siblings, and the PCR fragments were sequenced directly.

To genotype *vox*^{st9}, a *vox* DNA fragment was amplified with PCR primers 5′-CTATATAATCAGGCCGCTTTTGGAGACC-3′ and 5′-TTTCTCCGGCTCCTGGACATCCAG-3′ and digested with *Nla*III. *Df*^{st7} was scored by the presence or absence of *vent* (using the same primers as for meiotic mapping) or SSLP marker Z10362. Methods for genotyping *boz*^{m168}, *chd*^{tt250}, and *swr*^{ta72}, previously identified mutations used in this study, have been described (Fekany et al., 1999; Hild et al., 1999; Dick et al., 2000).

In situ hybridization

Probe synthesis and in situ hybridization were carried out as described (Sirotkin et al., 2000). For genotyping after photography, genomic DNA was extracted as described (Sirotkin et al., 2000).

Plasmid constructs

To construct the wild-type *vox* and *vox*^{st9} expression constructs, pCS2-vox and pCS2-vox-GTG, the coding region of the *vox* cDNA (Melby et al., 2000) was amplified by PCR and cloned into pCS2+ using oligonucleotides, 5′-CTG*GGATCC*ATCATGGTGAAGAACTTTTC-CGTG-3′ (*vox*) and 5′-CTG*GGATCC*ATCGTGGTGAAGAACTTT-TCCGTG-3′ (*vox*^{st9}), for sense primers, and 5′-AGG*CTCGAG*TCA-GTAGTAATGATGTCTGGGCATC-3′ for antisense primer. The italicized nucleotides are restriction sites used for cloning the PCR products and the underlined nucleotides are the initiation codon (ATG) and its mutant version (GTG). The insert sequences of these plasmids were confirmed by DNA sequencing.

RNA and morpholino oligonucleotide microinjections

To make *vox*, *vox*^{st9} and *vent* mRNA, pCS2-vox, pCS2-vox-GTG and ZV86 (see above and Melby et al., 2000) were linearized and transcribed with SP6 polymerase using the mMessage mMachine kit (Ambion). Morpholino oligonucleotides were obtained from Gene Tools LLC. Antisense *vent* morpholino (5′-CCACTGAGAACTTG-CTGGGTATCAT-3′), antisense *vox* morpholino (5′-AGTCCACGG-AAAAGTTCTTCACCAT-3′) and standard control morpholino (5′-CCTCTTACCTCAGTTACAATTTATA-3′) were used. RNA and morpholino oligonucleotides were diluted in 5 mg/ml Phenol Red, 0.2 M KCl prior to microinjection. 100 pl of RNA or morpholino oligonucleotide was microinjected into 1-8-cell stage embryos that had been dechorionated by Pronase treatment.

RESULTS

Isolation of new dorsalizing mutations

In a genetic screen for zebrafish mutants with early patterning defects, we isolated two new mutations that disrupt the development of the dorsoventral axis (see Materials and Methods). These mutations, *T(LG13)*^{st7} and *T(LG13)*^{st8}, strongly expand expression of the dorsal mesoderm marker *gsc* and produce a dorsalized phenotype. Although these mutations are translocations (see Materials and Methods), in both cases we were able to obtain unbalanced translocation heterozygotes that transmit simple Mendelian deletions (see below), which are called *Df(LG13)*^{st7} and *Df(LG13)*^{st8}. Because it is more rewarding to analyze these Mendelian mutations than balanced translocations, *Df(LG13)*^{st7} and *Df(LG13)*^{st8} were used for most of the experiments described below. For simplicity, we refer to these mutations as *Df*^{st7} and *Df*^{st8} hereafter.

Homozygotes for the *Df*^{st7} mutation have a marked truncation of the body axis at about 28 hours postfertilization (hours) (Fig. 1B; Table 1). In some crosses, *Df*^{st7} mutants have an elongated shape at the end of gastrulation and lyse during somitogenesis (data not shown). In addition to these zygotic effects, the *Df*^{st7} mutation has a weak dominant maternal effect (Fig. 1D; Table 1), at least in some genetic backgrounds. Following the classification of Mullins et al. (Mullins et al., 1996), in which dorsalized phenotypes range from class 1 (C1, weak) to class 5 (C5, strong), *Df*^{st7} homozygotes display C4/C5 dorsalized phenotypes. *Df*^{st8} mutants and *Df*^{st7}/*T*^{st8} transheterozygotes also have a C4/C5 dorsalized phenotype (Fig. 1E; Table 1). Because these results suggest that *Df*^{st7} and *Df*^{st8} delete at least some of the same essential genes, we have used *Df*^{st7} for most of our phenotypic studies. In stocks derived from crosses to the TL strain (Haffter et al., 1996), the *Df*^{st7} dorsalized phenotype is fully penetrant (Table 1). In other genetic backgrounds, the *Df*^{st7} mutation displays lower

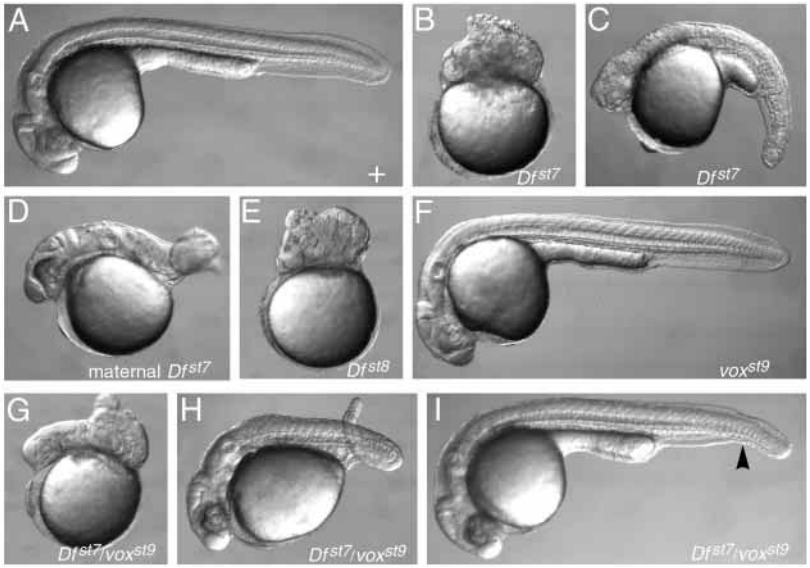

Fig. 1. Morphological analysis of dorsalized mutants. Pictures of live embryos at approximately 28-32 hours. (A) Wild type. (B) C4 dorsalized Df^{st7} mutant. (C) Extensive degeneration in Df^{st7} mutant in TL/WIK hybrid background. (D) C3 dorsalized embryo from cross of wild-type male and Df^{st7}/+ female. (E) C4 dorsalized Df^{st8} mutant. (F) Most homozygous vox^{st9} embryos have a wild-type appearance. (G-I) Df^{st7}/vox^{st9} mutants have variable phenotype, ranging from wild-type to strongly dorsalized. C4 (G), C3 (H) and C1 (I) phenotypes are shown. Arrowhead in I indicates reduced ventral tail fin.

penetrance and much more variable expressivity, such that some mutants do not have a dorsalized morphology (e.g. TL/WIK; Table 1). There is also an effect of maternal age on the maternal and zygotic effects of Df^{st7}; progeny of older females tend to have more severe phenotypes than progeny of younger females (data not shown). In backgrounds with a low frequency of dorsalized mutants, Df^{st7} homozygotes display extensive cell death at about 1 day after fertilization (Fig. 1C; Table 1). Regardless of the background and the extent of dorsalization, most Df^{st7} mutants display extensive cell death and degeneration by 30 hours (not shown). These results indicate that the region deleted in Df^{st7} contains loci essential for embryogenesis, including a gene or genes required for development of the dorsoventral axis.

Df^{st7} and Df^{st8} mutations delete a region of LG13 that includes *vox* and *vent*

As a first step to identify the critical gene(s) inactivated by Df^{st7} and Df^{st8}, we mapped the mutations. Analysis of simple-sequence length polymorphism (SSLP) markers (Shimoda et al., 1999) indicated that segments of LG13 are deleted in Df^{st7} and Df^{st8} mutants (Fig. 2A and data not shown). As shown in Fig. 2B, the Df^{st7} mutation stretches over at least 29 cM from SSLP marker Z17223 to Z4252, whereas the Df^{st8} mutation is somewhat longer, spanning a 35 cM interval defined by markers Z9423 and Z4252. The finding that Df^{st7} and Df^{st8} remove overlapping segments of LG13 supports the conclusion that these mutations inactivate the same gene(s) with crucial function in dorsoventral patterning. Furthermore, the mapping studies indicate that the Df^{st7} and Df^{st8} mutations do not inactivate *bmp2b*/*swr*, *bmp7*/*snh* or *smad5*/*sbn*, because these genes reside at different locations (LG20, LG11 and LG14, respectively).

Analysis of another LG13 rearrangement, $Df(LG13)^{st4}$ (Df^{st4} hereafter), was useful in defining the region of LG13-containing genes with important functions in dorsoventral patterning. In previous work (Imai et al., 2000), we showed that Df^{st4} deletes approximately 20-27 cM of LG 13, covering an interval that overlaps extensively but not completely with the region deleted in Df^{st7} and Df^{st8} (Imai et al., 2000; Fig. 2B). These three mutations were recovered in the same screen (Imai et al., 2000; this work), and they have been maintained on similar genetic backgrounds, suggesting that the genes affected by the rearrangements rather than genetic background are responsible for phenotypic differences among these mutants. Df^{st4}, Df^{st4}/Df^{st7} and Df^{st4}/Df^{st8} mutants are not

Table 1. Phenotypes produced by mutant crosses

Female	Male	Genetic background	Temperature (ºC)	Age (months)	Wild type (%)	C1 (%)	C2 (%)	C3 (%)	C4/C5 (%)	Degeneration by 24 hours (%)	*n*
$Df(LG13)^{st7}$/+	$Df(LG13)^{st7}$/+	TL	28		67.8	3	3	1	26	0	345
$Df(LG13)^{st7}$/+	$Df(LG13)^{st7}$/+	TL/WIK	28		71.9	0.5	0	0	8.1	19	395
$Df(LG13)^{st7}$/+	+/+	TL	28		92.1	2.0	1	2.0	3.1	0	833
+/+	$Df(LG13)^{st7}$/+	TL	28		100	0	0	0	0	0	429
$T(LG13)^{st8}$/+	$Df(LG13)^{st7}$/+	TL	28		90	0	0	0	10	0	228
$Df(LG13)^{st4}$/+	$Df(LG13)^{st7}$/+	TL	28		68	0	0	0	0	32	100
vox^{st9}/+	$Df(LG13)^{st7}$/+	TL	28	3-5*	97.7	1.7	0	0.5	0.2	0	1030
		TL	31	3-5	94.9	0.92	0.92	2.1	1.1	0	1086
		TL	28	9-10*	86.4	1	2	8.7	2	0	516
vox^{st9}/+	vox^{st9}/+	TL	28	3-5*	100	0	0	0	0	0	239
		TL	28	9-10*	97	1	0.5	1	0.3	0	627
vox^{st9}/+	+/+	TL	28	9-10	100	0	0	0	0	0	897
vox^{st9}/vox^{st9}	vox^{st9}/vox^{st9}	TL	28	3-5	100	0	0	0	0	0	311

*The same stocks of fish were used for these crosses at different times, minimizing differences due to genetic background.

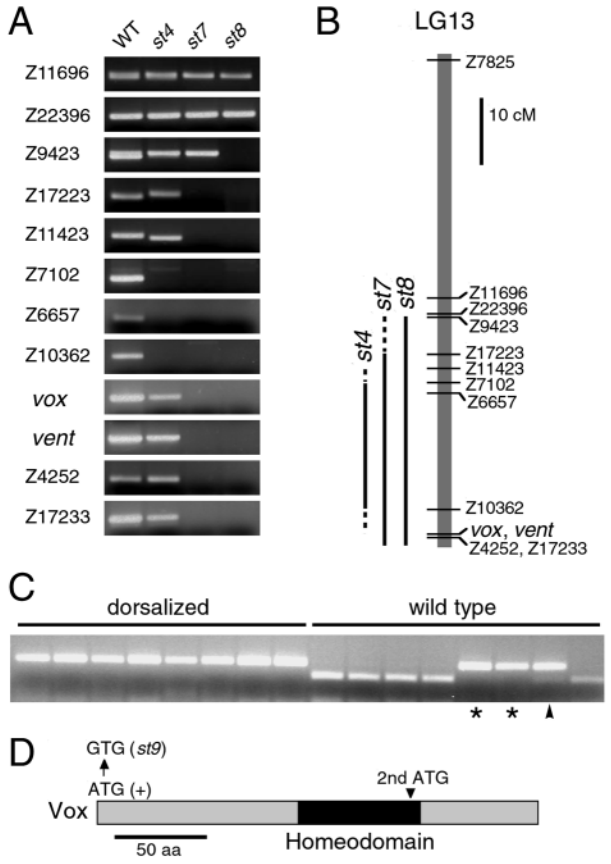

Fig. 2. Mapping and molecular analysis of Df^{st7}, Df^{st8} and vox^{st9}. (A) Genomic DNA from pools of wild type, Df^{st7}, Df^{st8} and Df^{st4} embryos were tested with LG13 SSLP markers and primers for *vox* and *vent*. These mutations remove distinct but overlapping sets of markers on LG13. All markers were also tested in individual embryos to confirm the presence or absence of the PCR products (data not shown). (B) Linkage map of LG13 showing the positions of *vox* and *vent* and the regions deleted in Df^{st7}, Df^{st8} and Df^{st4} (vertical lines indicate deleted regions, broken vertical lines denote uncertainty in the locations of the breakpoints). Marker distances are based on Shimoda et al. (Shimoda et al., 1999). We localized *vox* and *vent* by scoring polymorphisms in both genes in the HS meiotic mapping panel (Kelly et al., 2000). (C) The vox^{st9} mutation is tightly linked to *vox*. All dorsalized embryos (n=291) had only the mutant *vox* allele, indicating that their genotype was Df^{st7}/vox^{st9}. Three different genotypes were observed in embryos with a wild-type phenotype: only the wild-type allele (+/+ or Df^{st7}/+), both wild-type and mutant alleles (vox^{st9}/+; arrowhead), and only the mutant allele (Df^{st7}/vox^{st9}; asterisk). The Df^{st7}/vox^{st9} genotype is only partially penetrant, because some of these embryos have a wild-type phenotype. Analysis of SSLP markers confirmed that the vox^{st9} mutation resides in the distal region of LG13 (data not shown). (D) Schematic representation of Vox protein and the lesion in the vox^{st9} mutation. The vox^{st9} mutation changes the initiation codon (ATG) of the *vox* open reading frame to GTG. The second in-frame ATG occurs at codon 170, near the end of the homeodomain.

dorsalized (Imai et al., 2000; data not shown), suggesting that the gene(s) whose deletion is responsible for the dorsalized phenotype resides in the region deleted in Df^{st7} but not in Df^{st4}.

By scoring polymorphisms in our meiotic mapping panel (Kelly et al., 2000), we localized two candidate genes, the ventrally expressed homeobox genes *vox* and *vent* (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000), to the distal region of LG13, within the interval deleted in Df^{st7} but not Df^{st4} mutants (Fig. 2B). These results show that *vox* and *vent* reside in the region of LG13 that contains a gene or genes with important functions in dorsoventral patterning.

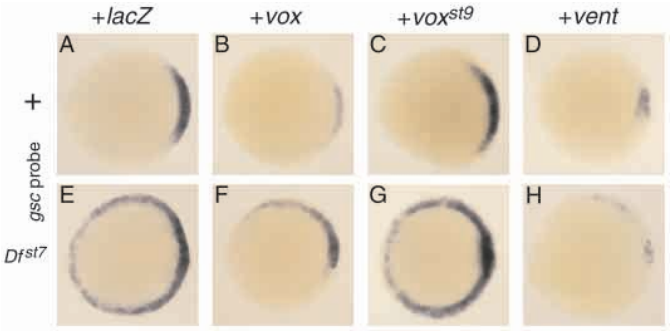

Fig. 3. Overexpression of *vox* or *vent* but not vox^{st9} can counteract the expansion of dorsal mesodermal fates in Df^{st7} mutants. Expression of *gsc* in wild-type (+) and Df^{st7} embryos injected with synthetic mRNA for *lacZ*, *vox*, vox^{st9} or *vent*. (A,E) 50 pg *lacZ*, (B,F) 50 pg *vox*, (C,G) 50 pg vox^{st9} or (D,H) 30 pg *vent* mRNA was injected into 1-4-cell stage embryos from Df^{st7}/+ intercrosses. Embryos were fixed at 40% epiboly (5 hours) and expression of *gsc* was examined by in situ hybridization. In clutches injected with *lacZ* (n=36) or vox^{st9} (n=41), *gsc* expression was normal in about three-quarters of the embryos and strongly expanded in about a quarter of the embryos. In contrast, no embryos had strongly expanded *gsc* expression in clutches injected with *vox* (n=38) or *vent* (n=54); *gsc* expression was weakly or partially expanded in about 13% (*vent*) or 16% (*vox*) of these embryos, while expression was normal or reduced in the other embryos of these clutches. In B,D,F,H, genotypes (+ or Df^{st7}) were determined by PCR assay after the photographs were taken. Animal pole views, dorsal towards the right. Similar results were obtained using *chd* expression to monitor the expansion of dorsal fates (data not shown).

Isolation of a point mutation in *vox*

To identify point mutations in genes responsible for the dorsalized phenotype of the deletions, we conducted a screen to find new mutations that fail to complement Df^{st7} (see Materials and Methods). In crosses of 448 F_1 females to Df^{st7}/+ males, we identified one mutation, vox^{st9}, that failed to complement Df^{st7}. A variable fraction of progeny (2-14%) from crosses of vox^{st9}/+ females to Df^{st7}/+ males had C1 to C4 dorsalized phenotypes (Fig. 1G-I), but many Df^{st7}/vox^{st9} individuals are wild-type in appearance (Table 1). The penetrance and expressivity of the Df^{st7}/vox^{st9} genotype are stronger at 31°C than 28°C (Table 1), as was shown by scoring the phenotypes of embryos from the same clutches incubated at the two temperatures. Most vox^{st9} homozygotes display no mutant phenotype and can grow to fertile adults (Fig. 1F; Table 1; see also Fig. 5B,J), but a variable dorsalized phenotype was apparent in some vox^{st9} homozygotes that were progeny of older females (Table 1). We noted a similar effect of maternal age on the penetrance and expressivity of the Df^{st7}/vox^{st9} genotype. An effect of maternal age has previously been noted in the analysis of *boz* mutants, which have more severe phenotypes when they are produced by young females and relatively less severe phenotypes when produced by older females (Fekany et al., 1999).

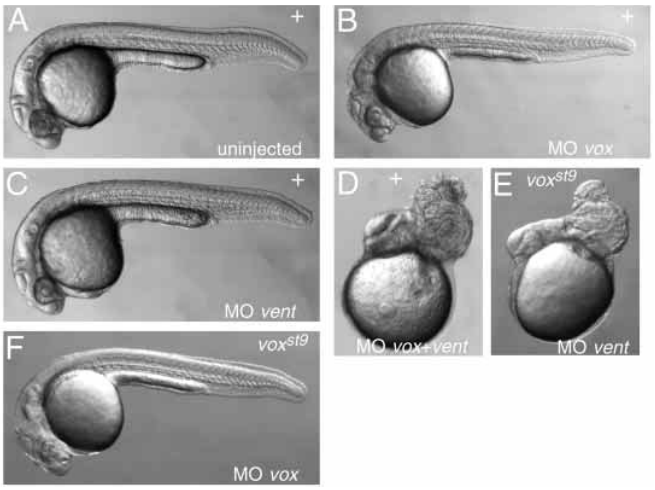

Fig. 4. Dorsalized phenotype of embryos lacking *vox* and *vent* function, owing to morpholino oligonucleotide injection. (A) Uninjected wild-type control embryo. Wild-type (B-D) or vox^{st9} (E,F) embryos were injected at the 1-4-cell stage with 840 pg *vox* antisense MO (B,F), 840 pg *vent* MO (C,E), or co-injected with 420 pg *vox* and 420 pg *vent* MO (D). Photographs of living embryos were taken at approximately 30 hours. Lateral views, anterior towards the left.

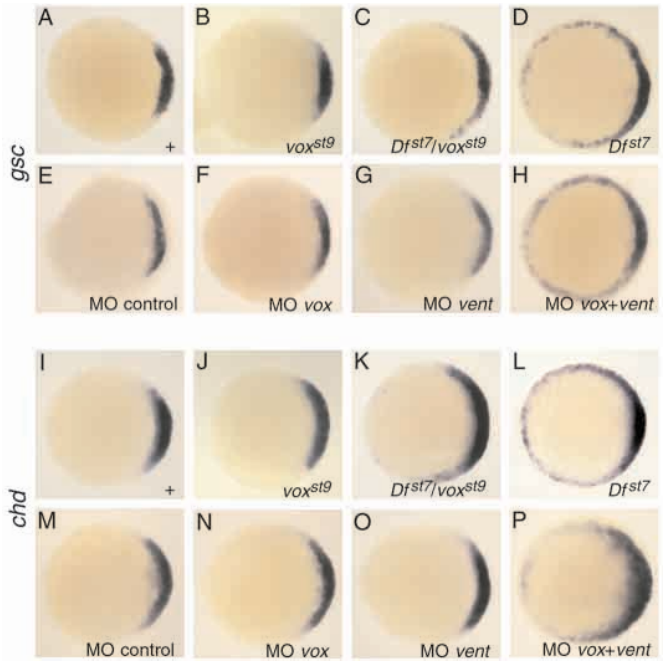

Fig. 5. Expression of *goosecoid* and *chordin* is ventrally expanded in embryos lacking *vox* and *vent* function. Expression of *gsc* (A-H) and *chd* (I-P) at 40% epiboly (5 hours) was examined by whole-mount in situ hybridization in wild type (A,I), vox^{st9} mutants (B,J), Df^{st7}/vox^{st9} mutants (C,K), Df^{st7} mutants (D,L), control MO-injected embryos (E,M), *vox* MO-injected embryos (F,N), *vent* MO-injected embryos (G,O), and in embryos co-injected with *vox* and *vent* MO (H,P). In B,C,J,K, genotypes were determined by PCR assay after the photographs were taken. Animal pole views, dorsal towards the right.

Mapping, molecular analysis, and functional studies demonstrated that the vox^{st9} mutation reduces or eliminates *vox* function. Genetic linkage analysis mapped vox^{st9} to the distal region of LG13, near *vox* and *vent* (Fig. 2B,C; data not shown). To search for a possible lesion, we sequenced the coding sequences of *vox* and *vent* from vox^{st9} mutants. Although no lesions were detected in the *vent* open reading frame, the *vox* gene contained an A→G change that disrupted the translational initiation codon (ATG→GTG; Fig. 2D). This ATG→GTG change was not present in genomic DNA (prepared from tail fin) of the mutagenized male (G_0) that gave rise to the vox^{st9} mutation, or in genomic DNA from 8 F_1 siblings of the original F_1 $vox^{st9}/+$ heterozygote (not shown). These results indicate that the parental chromosomes did not contain the ATG→GTG change, and that, instead, this mutation was induced by ENU in the germline of the mutagenized male. Using a restriction enzyme test for the A→G change, we found that all dorsalized Df^{st7}/vox^{st9} mutants (n=291) inherited the A→G mutation, demonstrating that this lesion is tightly linked to vox^{st9} (Fig. 2C). The second in-frame ATG in the *vox* sequence occurs 170 codons downstream of the wild-type translational start site, near the end of the homeodomain, suggesting that the vox^{st9} mutation results in an N-terminally truncated protein with little or no activity. To test the effect of the mutation on *vox* function, we microinjected wild-type embryos with either synthetic mRNA from a wild-type *vox* construct or mRNA transcribed from a *vox* construct engineered to contain the ATG→GTG change. More than 40% (n=61) of embryos injected with 50 pg of wild-type *vox* mRNA had reduced forebrain and/or notochord (classified as V1-V3 according to Kishimoto et al., 1997), while a *vox* mRNA engineered to contain the vox^{st9} lesion had no detectable ventralizing activity in this assay (100% wild-type, n=58). These results indicate that the vox^{st9} allele results in a strong or complete loss of *vox* function.

The finding that a mutation in *vox* failed to complement Df^{st7} suggested that loss of *vox* function contributes to the dorsalized phenotype of Df^{st7} mutants. To determine whether expression of *vox* could rescue the dorsalized phenotype of the deletion mutants, we injected synthetic *vox* mRNA into embryos from $Df^{st7}/+$ intercrosses and assayed *gsc* (Fig. 3) and *chd* (data not shown) expression at the late blastula stage (40% epiboly, 5 hours). In wild-type embryos at this stage, *gsc* is expressed specifically in dorsal mesendodermal precursors, and *chd* is expressed more broadly, in a dorsal domain that includes cells in presumptive neural plate and paraxial mesoderm in addition to axial mesendoderm (Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997). In Df^{st7} mutants injected with a control RNA (Fig. 3E; data not shown), *gsc* and *chd* expression was strongly expanded, such that transcripts were detected in the ventral marginal region. Overexpression of *vox* reduced the expansion of *gsc* and *chd* in Df^{st7} mutants (Fig. 3F; data not shown), and, consistent with previous studies (Kawahara et al., 2000a; Melby et al., 2000), also reduced the domains of expression in wild-type embryos (Fig. 3B; data not shown). Overexpression of *vent* also reduced the expanded domains of *gsc* and *chd* in Df^{st7} mutants (Fig. 3H; data not shown) and reduced the dorsal domains of *gsc* and *chd* expression in wild-type embryos (Fig. 3D; data not shown). Synthetic *vox* RNA with the vox^{st9} ATG→GTG mutation had no detectable activity in this assay (Fig. 3C,G). These results demonstrate that *vox* and *vent* have similar ability to counteract the expansion of dorsal gene expression in Df^{st7} mutants.

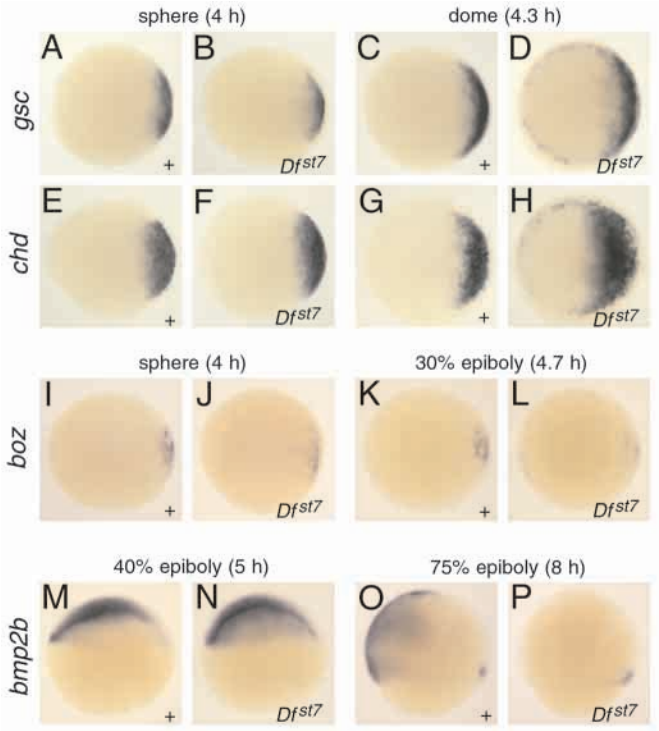

Fig. 6. Asymmetry of dorsal and ventral gene expression is established but not maintained in Df^{st7} mutants. Expression of *gsc* (A-D), *chd* (E-H), *boz* (I-L) and *bmp2b* (M-P) was examined by in situ hybridization at the indicated stages in wild-type and Df^{st7} mutants (genotypes shown at the bottom of each panel). In A,B,E,F,I,J,M,N, genotypes were determined by PCR assay after the photographs were taken. (A-L) Animal pole views, dorsal towards the right. (M-P) Lateral views, dorsal towards the right. We obtained similar results to those shown in N and P when we examined *bmp2b* expression in wild-type embryos co-injected with *vox* and *vent* MO.

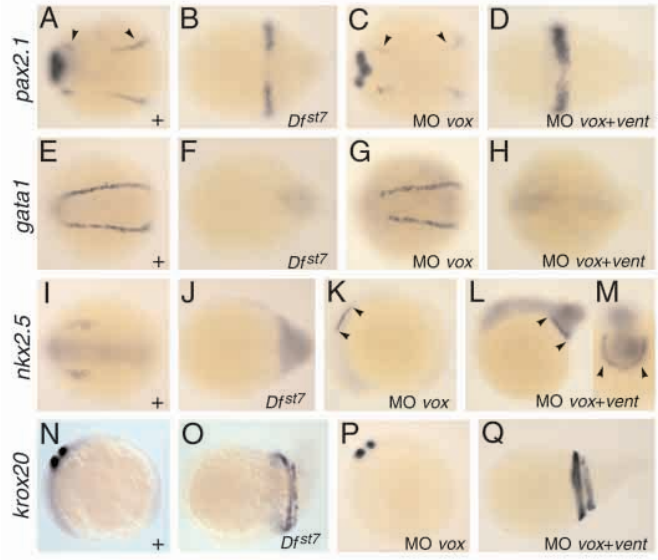

Fig. 7. Loss of ventrolateral mesoderm and expansion of neural territories in embryos that lack *vox* and *vent* function. Expression of *pax2.1* (A-D), *gata1* (E-H), *nkx2.5* (I-M) and *krox20* (N-Q) was examined by in situ hybridization at the (A-D,N-Q) three-somite stage (11 hours) or (E-M) 9-10 somite stage (~14 hours). (A,E,I,N) Wild type. (B,F,J,O) Df^{st7} mutants. (C,G,K,P) Wild-type embryos injected with *vox* MO. (D,H,L,M,Q) Wild-type embryos co-injected with *vox* and *vent* MO. (A-D) *pax2.1* is expressed in the pronephros and pronephric duct (arrowheads in wild-type embryos) and at the mid-hindbrain junction. (K-M) Arrowheads mark expression of *nkx2.5*. (A-J) Dorsal views, anterior towards the left. (K,L,N-Q) Lateral views, anterior towards the left. (M) Posterior view, dorsal towards the top.

Dorsalized phenotype results from inactivation of *vox* and *vent*

The overexpression experiments, together with the analysis of the vox^{st9} mutation support the idea that the loss of *vox* function contributes to the dorsalized phenotype of Df^{st7} homozygotes. *vox*, however, cannot be the only important gene removed by the deletion, because Df^{st7}/vox^{st9} mutants have a weaker and more variable phenotype than Df^{st7} homozygotes, and most vox^{st9} homozygotes are not detectably dorsalized (Fig. 1; Table 1). Because *vox* and *vent* have similar ventralizing activities in overexpression assays (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000; Fig. 3) and because both *vox* and *vent* are deleted in Df^{st7} mutants, we hypothesized that *vox* and *vent* have overlapping activities, such that both genes must be inactivated to produce a strong dorsalized phenotype.

Table 2. Dorsalization of embryos by injection of *vox* and *vent* morpholino oligonucleotides

Strain	Morpholino	Dose	Wild type (%)	C1 (%)	C2 (%)	C3 (%)	C4-C5 (%)	*n*
Wild type (TL)	control	840 pg	100	0	0	0	0	81
	vox	840 pg	94	5	2	0	0	62
	vent	840 pg	93	2	3	2	0	61
	vox+vent	420 pg+420 pg	0	0	0	0	100	80
vox^{st9}/+ (TL)	control	840 pg	100	0	0	0	0	58
	vox	840 pg	98	2	0	0	0	53
	vent	840 pg	74	1	0	2	22*	81
	vox+vent	420 pg+420 pg	0	0	0	0	100	29
Wild type (AB)	control	840 pg	100	0	0	0	0	49
	vox	840 pg	100	0	0	0	0	56
	vent	840 pg	98	2	0	0	0	47
	vox+vent	420 pg+420 pg	91	6	3	0	0	35

*Five of these strongly dorsalized embryos were genotyped by PCR assay: all were vox^{st9} homozygotes.

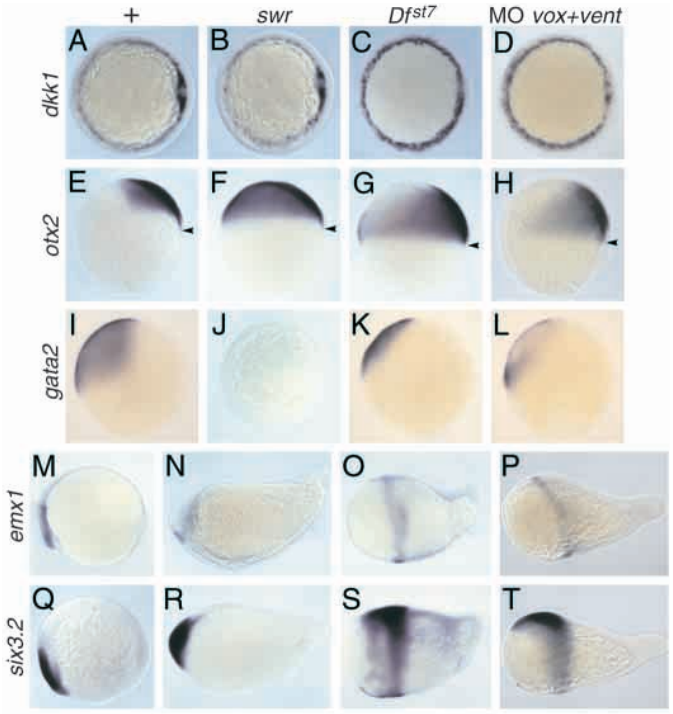

Fig. 8. Distinct neural patterning phenotypes in *bmp2b*/*swr* mutants and embryos lacking *vox* and *vent* function. Expression of *dkk1* (A-D), *otx2* (E-H), *gata2* (I-L), *emx1* (M-P) and *six3.2* (Q-T) was examined by in situ hybridization in wild-type (A,E,I,M,Q), *swr* mutant (B,F,J,N,R), Df^{st7} mutant (C,G,K,O,S) embryos and MO *vox* + *vent* embryos (D,H,L,P,T). Arrowheads in E-H mark the vegetal limit of *otx2* expression. (A-D) Shield stage (6 hours), animal pole views, dorsal towards the right. (E-L) 75% epiboly (8 hours), lateral views, dorsal towards the right. (M-T) three-somite stage (11 hours), lateral views, anterior towards the left. In A,B, genotypes were determined by PCR assay after the photographs were taken.

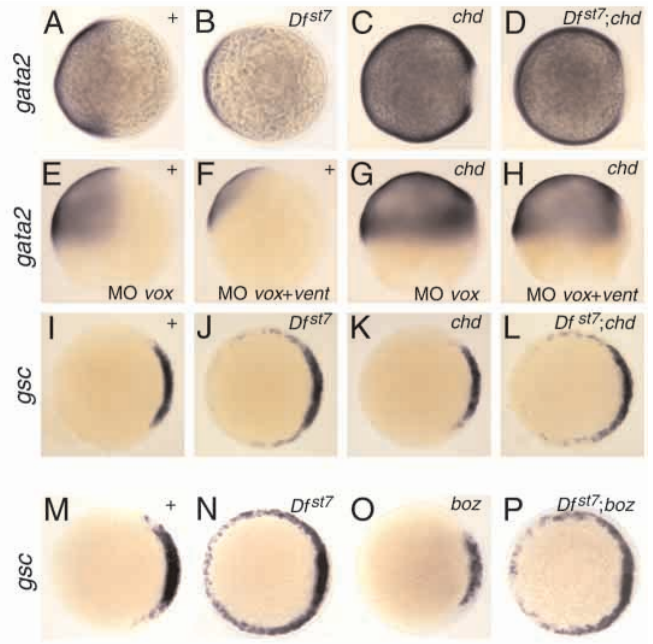

Fig. 9. Interactions among *vox*, *vent*, *chordin* and *bozozok*. The genotype of the embryos is shown at the top of the panel, and the use of MO, when injected, is shown at the bottom of the panel. The gene analyzed by in situ hybridization is shown on the left side of each row. Embryos were analyzed at 75% epiboly (A-H), 40% epiboly (I-L), or 50% epiboly (M-P). In *boz* mutant embryos co-injected with *vox* and *vent* MO, *gsc* expression resembled that shown in P (data not shown). In Df^{st7};*chd* double mutants, domains of *gata2* (D) and *gsc* (L) expression are both expanded when compared with wild type (A,I). In each of several cases examined, *gata2* expression was slightly less expanded in Df^{st7}; *chd* mutants (D) than in *chd* single mutants (C); a similar result was observed in comparing *chd* mutants injected with *vox* and *vent* MO (H) and *chd* mutants injected with *vox* MO (G). The reduced expansion of the ventral marker *gata2* (D,H) correlates with the increase in *gsc* expression in *chd* mutants lacking *vox* and *vent* function (L). (A-D,I-P) Animal pole views, dorsal towards the right. (E-H) Lateral views, dorsal towards the right. In A-D,I-P, genotypes were determined by PCR assay after the photographs were taken.

As another approach to examine the phenotypic effects of reducing *vox* and *vent* function, we employed antisense morpholino oligonucleotides (MO). In *Xenopus* and zebrafish, microinjection of MO complementary to the region of the translational initiation codon or 5′ untranslated region can reduce protein levels and produce specific loss-of-function phenotypes (Heasman et al., 2000; Nasevicius and Ekker, 2000). Injection of 1-8-cell wild-type (TL strain) embryos with 840 pg (0.1 pmol) of antisense MO for either *vox* or *vent* individually produced only a low frequency of weakly dorsalized embryos (Fig. 4B,C; Table 2). In contrast, co-injection of wild-type (TL strain) embryos with 420 pg of *vox* MO and 420 pg of *ven*t MO produced a strong dorsalized phenotype at very high frequency (Fig. 4D; Table 2). Co-injection of embryos from a different wild-type strain (AB) with *vox* and *vent* MO produced weaker and more variable phenotype (Table 2), reminiscent of the effect of genetic background on the phenotype of Df^{st7} mutants (Table 1). As a control for the specificity of the MO, we injected embryos from intercrosses of vox^{st9}/+ heterozygotes with the *vox* and *vent* MO, individually or in combination. Although injection of 840 pg of *vent* MO had a very weak effect on wild-type embryos (Fig. 4C; Table 2), this treatment strongly dorsalized vox^{st9} mutants (Fig. 4E; Table 2). Injection of 840 pg of *vox* MO had little effect on vox^{st9} mutants (Fig. 4F; Table 2), and co-injection of *vox* and *vent* MO dorsalized vox^{st9} mutants, just as with wild-type embryos (Table 2). These experiments demonstrate *vox* and *vent* have redundant functions and that the loss of both *vox* and *vent* produces a strong but strain-dependent dorsalized phenotype.

Loss of *vox* and *vent* function expands dorsoanterior cell types

To determine what cell types are affected by the loss of *vox* and *vent* function, we examined marker gene expression in Df^{st7} homozygotes and wild-type embryos co-injected with *vox* and *vent* MO (which we refer to as 'MO *vox* + *vent* embryos'). For these experiments, a TL-derived genetic background was used, in which the Df^{st7} mutation produces a fully penetrant C4-C5 dorsalized phenotype (Table 1). Similarly, the MO injections were conducted with a wild-type strain (TL) in which the co-injection produced a strongly dorsalized morphological phenotype at very high frequency

(Table 2). Although one marker revealed phenotypic differences between Df^{st7} and MO *vox* + *vent* embryos (see below), overall these phenotypes were very similar.

Assays of marker gene expression revealed that *vox* and *vent* are required for proper patterning of the dorsoventral axis in the late blastula. In both Df^{st7} mutants and MO *vox* + *vent* embryos at the late blastula stage (40% epiboly, 5 hours), expression of *gsc* (Fig. 5D,H) and *chd* (Fig. 5L,P) was strongly expanded. The expression domains of *flh* and *axial*, two other dorsally restricted genes, were also expanded in Df^{st7} mutants during gastrulation (data not shown). Consistent with the morphological phenotypes at later stages, *gsc* and *chd* are expressed normally in wild-type embryos injected with either *vox* MO (Fig. 5F,N) or *vent* MO (Fig. 5G,O) and in vox^{st9} embryos (Fig. 5B,J); partially expanded domains were observed in some Df^{st7}/vox^{st9} mutants (Fig. 5C,K). At an earlier stage (sphere stage, 4 hours), Df^{st7} mutants appear to have normal expression of the dorsally restricted genes *gsc*, *boz*, and *chd* (Fig. 6B,F,J). Slightly later (dome stage, 4.3 hours), however, Df^{st7} mutants have a pronounced expansion of *gsc* and *chd* expression (Fig. 6D,H), such that transcripts were detected in some ventral marginal cells. At dome stage, *boz* was expressed in its normal dorsal domain in Df^{st7} mutants (data not shown), but at 30% epiboly (4.7 hours) *boz* expression was expanded (Fig. 5L). These results indicate that the redundant action of *vox* and *vent* is required for maintenance, but not initiation, of spatially restricted dorsal gene expression in the late blastula.

The function of *vox* and *vent* is also required for proper expression of ventrally restricted genes during gastrulation. As in wild type, *bmp2b* and *bmp4* are strongly expressed in the ventral but not dorsal regions of Df^{st7} mutants (Fig. 6N) and MO *vox* + *vent* embryos (data not shown) at the late blastula stage (40% epiboly, 5 hours). At the mid-gastrula stage (75% epiboly, 8 hours), however, ventral expression of *bmp2b* and *bmp4* was strongly reduced in Df^{st7} mutants and MO *vox* + *vent* embryos (Fig. 6P and data not shown). Similarly, the ventral marker *gata2* was reduced at the mid-gastrula stage (75% epiboly, 8 hours), but it was expressed normally at the start of gastrulation in Df^{st7} mutants and in MO *vox* + *vent* embryos (see Figs 8K,L, 9B,F; data not shown). Thus, the redundant action of *vox* and *vent* is required for maintenance of ventral gene expression at the mid-gastrula stage, but *vox* and *vent* are not required for proper expression of ventral markers at earlier stages.

To investigate whether *vox* and *vent* function in the development of ventrolateral mesodermal derivatives, we examined markers of pronephros, blood, and heart in Df^{st7} mutants and MO *vox* + *vent* embryos (Fig. 7). Analysis of *pax2.1* expression revealed that the developing pronephros, a derivative of intermediate mesoderm, was absent in Df^{st7} mutants and MO *vox* + *vent* embryos (Fig. 7B,D). Similarly, expression of the erythroid lineage marker *gata1* was not detected (Fig. 7F,H), indicating that *vox* and *vent* are essential for the development of blood. To investigate whether *vox* and *vent* function in development of the heart, we examined *nkx2.5*, a gene expressed in cardiac precursors. Expression was not detected in Df^{st7} mutants (Fig. 7J), but *nkx2.5* transcripts were evident in MO *vox* + *vent* embryos (Fig. 7L,M). Similarly, Df^{st7} mutants did not form a beating heart by approximately 30 hours, in contrast to MO *vox* + *vent* embryos, which did have a beating heart at this stage (data not shown). This difference between the cardiac phenotypes of Df^{st7} mutants and MO *vox* + *vent* embryos suggests that another gene within the Df^{st7} deletion may be required for heart development, or perhaps that *vox* + *vent* MO co-injection does not eliminate the functions of these genes in cardiac precursors.

Examination of neural markers shows that dorsoanterior territories are expanded in embryos that lack *vox* and *vent* function. Expression of *pax2.1* and *krox20*, which mark the midbrain-hindbrain boundary and hindbrain rhombomeres 3 and 5, respectively, was expanded to ventral regions in Df^{st7} mutants and MO *vox* + *vent* embryos (Fig. 7B,D,O,Q). The expression domains of these genes were also shifted posteriorly (Fig. 7B,D,O,Q), suggesting that more anterior territories are expanded in embryos lacking *vox* and *vent* function.

To investigate the possibility that BMP signals and Vox/Vent have different functions in neural patterning, we examined neural markers in *swr*/*bmp2b* and Df^{st7} mutants (Fig. 8). In wild-type embryos at the mid-gastrula stage, *otx2* is expressed in a dorsal animal domain that includes presumptive forebrain and midbrain territories. In *swr*/*bmp2b* mutants (Fig. 8F), *otx2* was expanded ventrally but not vegetally, such that the expression domain encompassed both dorsal and ventral regions, but the vegetal border was at the wild-type position (arrowhead in Fig. 8F). In Df^{st7} mutants and MO *vox* + *vent* embryos, *otx2* expression was expanded vegetally (arrowheads in Fig. 8G,H); expression was also expanded ventrally, but not to the same extent as in *bmp2b*/*swr* mutants. In Df^{st7} mutants and MO *vox* + *vent* embryos, the ventral (non-neural) ectoderm marker *gata2* was reduced from wild-type levels (Fig. 8K,L), and the expression domain was roughly complementary to that of *otx2* (compare Fig. 8G,H with 8K,L). As in previous studies (Nguyen et al., 1998), we did not detect *gata2* expression in *swr*/*bmp2b* mutants (Fig. 8J). Analysis of markers at later stages confirmed that anterior neural territories are expanded in Df^{st7} mutants and MO *vox* + *vent* embryos. The telencephalon marker *emx1* is strongly expanded in Df^{st7} mutants and MO *vox* + *vent* embryos (Fig. 8O,P), but not in *bmp2b*/*swr* mutants (Fig. 8N). Similarly, *six3.2*, a marker of forebrain, optic primordia and axial mesendoderm, was expressed much more strongly in Df^{st7} mutants and MO *vox* + *vent* embryos (Fig. 8S,T) than in wild-type (Fig. 8Q) and *swr*/*bmp2b* mutants (Fig. 8R).

The finding that anterior neural territories are enlarged in Df^{st7} mutants and in MO *vox* + *vent* embryos (Figs 7, 8) suggested that *vox* and *vent* may repress the expression of signals that induce anterior neural identity. Supporting this possibility, we found that Df^{st7} mutants and MO *vox* + *vent* embryos had greatly increased expression of *dkk1* (Fig. 8C,D), which encodes a secreted Wnt-antagonist with anteriorizing activity (Glinka et al., 1998; Hashimoto et al., 2000; Shinya et al., 2000). In *bmp2b*/*swr* mutants at the early gastrula stage (shield stage, 6 hours), *dkk1* was not expanded (Fig. 8B). These results indicate that the redundant action of *vox* and *vent* is required for the repression of anterior neural territories, and that *vox*/*vent* and *bmp2b*/*swr* have distinct functions in neural patterning.

Interactions between *vox*, *vent* and *chordin*: increased activity of *chordin* represses ventral gene expression in embryos that lack *vox* and *vent*

Our results show that *vox* and *vent* act redundantly to

antagonize *chordin* expression (Fig. 5). Hence, aspects of the dorsalized phenotype caused by the lack of *vox* and *vent* might result from ectopic expression of the dorsalizing factor *chordin*. To investigate this possibility, we examined the ventral marker *gata2* in Df^{st7};*chd* double mutants and in *chd* single mutants co-injected with *vox* and *vent* MO. *chd* mutants are ventralized, and *gata2* is expressed in all but the most dorsal cells in *chd* mutant embryos (Hammerschmidt et al., 1996a; Hammerschmidt et al., 1996b; Fig. 9C). In Df^{st7};*chd* double mutants (Fig. 9D) and in *chd* single mutants co-injected with *vox* and *vent* MO (Fig. 9H), *gata2* is strongly expanded, indicating that *chd* is epistatic to *vox* and *vent* when assayed with this marker. These results support the model that *vox* and *vent* act to promote ventral gene expression by antagonizing the action of *chd* (Fig. 10).

To determine whether the increased expression of dorsal mesodermal genes in embryos lacking *vox* and *vent* function is caused by expanded expression of *chd*, we examined *gsc* expression in Df^{st7};*chd* double mutants. Consistent with previous studies (Hammerschmidt et al., 1996a), *gsc* expression was normal in *chd* single mutants (Fig. 9K) at the late blastula stage (40% epiboly, 5 hours). In Df^{st7};*chd* double mutants (Fig. 9L), *gsc* transcripts were detected in lateral and ventral marginal cells, such that *gsc* expression was very similar in Df^{st7};*chd* and Df^{st7} mutant embryos (Fig. 9J). Thus mutation of *chd* did not affect *gsc* expression in embryos lacking *vox* and *vent* function, suggesting that *vox* and *vent* do not regulate *gsc* transcription via antagonism of *chd* action. Instead it is likely that the Vox and Vent proteins directly repress the transcription of *gsc* and *chd* (Melby et al., 1999; Trinidade et al., 1999; Kawahara et al., 2000b).

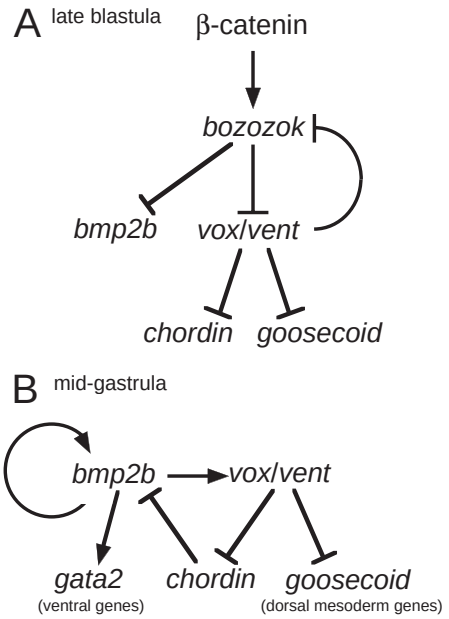

Fig. 10. Model of interactions between *vox*, *vent* and other genes that pattern the dorsoventral axis. (A) In the late blastula *boz* represses *vox*, *vent* and *bmp2b* in parallel. *vox* and *vent* act redundantly to repress *chd*, *gsc* and other genes expressed in dorsal mesendoderm. (B) During gastrulation, *bmp2b* activates expression of ventral genes, such as *gata2*, *bmp4* and *bmp2b* itself, and represses expression of neural genes in ventral ectoderm. By mid-gastrulation, *vox*/*vent* and *bmp2b* establish a positive feedback loop in which *bmp2b* activates expression of *vox* and *vent*, and *vox* and *vent* activate expression of *bmp2b* indirectly, via repression of *chd*.

Interactions between *vox*, *vent* and *boz*: increased activity of *vox* and *vent* represses dorsal mesoderm in *boz* mutants

We have shown that *boz* expression is expanded in embryos that lack *vox* and *vent* function (Fig. 6), and previous work shows that expression of *vox* and *vent* is expanded in *boz* mutants (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). To investigate the order in which these genes act, we examined *gsc* expression in embryos lacking the function of *vox*, *vent* and *boz*. Expression of *gsc* was reduced in *boz* mutants at the late blastula stage (Fig. 9O) – the opposite phenotype of Df^{st7} mutants (Fig. 9N). In Df^{st7};*boz* double mutants (Fig. 9P) and in *boz* single mutants that were co-injected with *vox* and *vent* MO (data not shown), *gsc* expression was expanded, indicating that *vox* and *vent* are epistatic to *boz*. Thus, *boz* function is not required to activate *gsc* expression at normal levels if *vox* and *vent* are inactivated by mutation or morpholino oligonucleotide injection. These results support a model in which *boz* promotes dorsal mesodermal gene expression by antagonizing the action of *vox* and *vent* (Fig. 10).

DISCUSSION

Redundant ventralizing functions of *vox* and *vent*

Our results demonstrate that the *vox* and *vent* homeobox genes have redundant functions in dorsoventral patterning in the zebrafish embryo. Evidence for this conclusion derives from our analysis of a point mutation that disrupts *vox*, chromosomal rearrangements that delete *vox*, *vent* and numerous other genes on LG13, and antisense morpholino oligonucleotides that inactivate *vox* and *vent*. Inactivation of either *vox* or *vent* by MO injection has little phenotypic effect on the embryo. Similarly, most homozygotes for the loss-of-function allele vox^{st9} are wild type in appearance and viable to adulthood. In contrast, embryos were strongly dorsalized when we inactivated both *vox* and *vent* by any of three approaches: co-injection of *vox* and *vent* MO into wild type, injection of *vent* MO into vox^{st9} homozygotes, or deletion of the *vox*/*vent* region of LG13 in Df^{st7} and Df^{st8} mutants. The combined results show that inactivation of either *vox* or *vent* has little effect, whereas the disruption of both genes produces a severely dorsalized phenotype. The phenotype of embryos that lack *vox* and *vent* function is strongly affected by genetic background. Our results suggest that the AB background has one or more dominant genes that suppress the dorsalized phenotype caused by loss of *vox* and *vent* function. Preliminary analysis suggests that the background effect may be due to one major gene in some crosses, but the penetrance in other crosses suggests that multiple genes may be responsible (Y. Imai, unpublished). Furthermore, the penetrance and expressivity are also strongly affected by maternal age, making it difficult to determine the number of the genes involved in the background effect. Further analysis is required to determine the nature of the genetic differences between strains that affect the phenotype caused by the loss of *vox* and *vent* function.

Consistent with our genetic evidence for the redundant functions of *vox* and *vent*, previous work shows that *vox*/*vent* family genes have similar activities in overexpression assays (Gawantka et al., 1995; Ault et al., 1996; Ladher et al., 1996; Onichtchouk et al., 1996; Onichtchouk et al., 1998; Schmidt et al., 1996; Melby et al., 1999; Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). *Vox*, *Xvent-1*, *vox* and *vent* can each ventralize the embryo and repress the expression of dorsal genes such as *chd* and *gsc* in overexpression assays. In addition, we found that injection of synthetic mRNA encoding either Vox or Vent could counteract the expansion of dorsal marker gene expression in Df^{st7} mutants, providing further evidence that function of either gene is sufficient to repress dorsal gene expression. Our results suggest that the differences between the activities of Vox and Vent are largely quantitative, and that both proteins are capable of repressing the same set of crucial target genes. Our results also suggest that the combined level of *vox* and *vent* activity is crucial for establishing normal dorsoventral pattern: vox^{st9} homozygotes have little or no dorsoventral patterning defect, Df^{st7} deletion homozygotes have a strongly dorsalized phenotype and Df^{st7}/vox^{st9} transheterozygotes, which lack *vox* function and have reduced *vent* function, have a range of phenotypes between these extremes.

vox and *vent* act in the marginal region to repress dorsal gene expression

Our results suggest that the requirement for *vox* and *vent* function is largely limited to the marginal cells. While *vox* expression encompasses all but the dorsal marginal cells of the late blastula, expression of *vent* is predominantly restricted to the ventrolateral marginal region (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). Despite the widespread expression of *vox*, our results indicate that the *vent* gene can compensate for the loss of *vox* in vox^{st9} homozygotes. Because the expression domains of these redundant genes overlap predominantly in marginal territories, the action of *vox* and *vent* is apparently not essential for the repression of dorsal genes in animal territories. Thus, other ventrally expressed transcription factors may limit neural genes to their proper dorsal ectodermal territories. One candidate for such a factor is *gata2*, expression of which is roughly complementary to *otx2* in wild type and in embryos that lack *vox* and *vent* function. Another candidate is a zebrafish counterpart of *Xenopus* Msx-1, which is a transcriptional repressor induced by ventralizing BMP signals (Yamamoto et al., 2000).

vox and *vent* redundantly repress *chordin* and other dorsal genes

Our loss-of-function analysis and previous overexpression assays (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000) show that many dorsally expressed genes, including *dkk1*, *squint*, *cyclops*, *flh*, *chd* and *gsc*, are repressed by Vox and Vent. Our genetic analysis indicates that *chd* is a key target of *vox* and *vent*. Expression of the ventral marker *gata2* is reduced in mid-gastrula stage embryos lacking *vox* and *vent* function and strongly expanded in *chd* mutant embryos. Expression of *gata2* is also strongly expanded in Df^{st7};*chd* double mutants, supporting the model that *vox* and *vent* activate *gata2* expression indirectly, via the inhibition of *chd* (Fig. 10). In *Xenopus*, Vox directly represses transcription of the *chd* gene (Melby et al., 1999), suggesting that Vox may act directly to inhibit *chd* expression in zebrafish. Interestingly, expression of the dorsal mesoderm marker *gsc* is expanded in Df^{st7};*chd* double mutants at the late blastula stage, indicating that the expansion of *gsc* expression in embryos lacking *vox* and *vent* function is not a consequence of expanded *chd* activity. Evidence in frog and zebrafish supports the possibility that Vox and Vent directly repress the transcription of *gsc* (Melby et al., 1999; Trindade et al., 1999; Kawahara et al., 2000b). Anterior neural territories are expanded in embryos that lack *vox* and *vent* function, possibly due to increased expression of *dkk1* or another dorsally expressed factor with anteriorizing activity. These results demonstrate that the redundant action of *vox* and *vent* is required to restrict expression of *chd* and other key dorsal genes to the appropriate territories.

Different roles of zygotic BMP signals and Vox/Vent in promoting development of ventral fates

Our results suggest that Vox/Vent homeodomain proteins and zygotic BMP signals play different roles in the establishment of ventral positional identity in the late blastula. We suggest that *vox* and *vent* function in the ventral marginal region to restrict the expression of the dorsal mesendodermal genes to the proper dorsal marginal territories, while the main role of zygotic BMP signals may be to activate ventral gene expression in ventrolateral territories (Fig. 10). This model is based on the following observations.

First, dorsal mesodermal fates are markedly expanded at early stages in embryos lacking *vox* and *vent* function, but not in *bmp2b*/*swr* mutants. In Df^{st7} mutants and wild-type embryos co-injected with *vox* and *vent* MO, genes characteristic of prospective dorsal mesoderm, including *gsc*, *chd* and *boz*, are expressed widely along the margin. In some cases, even the most ventral cells contain transcripts for these genes. In contrast, expression of *gsc* is normal in *swr* mutants at the early gastrula stage (Mullins et al., 1996). Thus, *vox* and *vent* act redundantly in the late blastula to restrict the expression of dorsal mesendodermal genes to the appropriate dorsal marginal territories. *bmp2b*/*swr* is not required for this restriction, indicating that the Vox and Vent repressors do not act simply as effectors of Bmp2b. Instead, we propose that Vox/Vent and zygotic BMP signals have distinct, partially parallel functions required for the development of ventrolateral mesendoderm. In the ventral marginal region, we suggest that *bmp2b* activates genes required for differentiation of ventrolateral mesodermal cell types, while the redundant action of *vox* and *vent* restricts dorsal mesendodermal genes to the proper dorsal marginal territories (Fig. 10).

Second, phenotypic differences in the neural development of embryos that lack *vox* and *vent* function and of *bmp2b*/*swr* mutants indicate that Vox/Vent and Bmp2b have different roles in patterning the ectoderm. During gastrulation, the dorsalization of the ectoderm is more complete in *bmp2b*/*swr* mutants than in embryos that lack *vox* and *vent* function. At mid-gastrulation in *bmp2b*/*swr* mutants, the ventral marker *gata2* is not expressed, and the dorsal marker *otx2* is strongly expressed even in ventral ectoderm. In contrast, *gata2* is expressed ventrally in embryos that lack *vox* and *vent* function, although at less than wild-type levels, and *otx2* expression is not expanded to the same extent as *bmp2b*/*swr* mutants. Thus, Bmp2b appears to be more important than Vox and Vent to

promote ventral gene expression and to restrict the expression of neural genes within the ectoderm, an activity that may be mediated by BMP-dependent ventral expression of transcription factors such as Gata2.

A positive feedback loop between Vox/Vent and BMP signals in the gastrula

vox/*vent* and *bmp2b*/*swr* are initially expressed independently of each other's action. In the late blastula, *bmp2b*/*swr* is expressed normally in embryos that lack *vox* and *vent* function, and *bmp2b*/*swr* mutants have normal expression of *vox* and *vent* (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). As embryogenesis proceeds, however, expression of *vox*/*vent* and *bmp2b*/*swr* genes becomes interdependent, apparently through a positive feedback loop established during gastrulation (Fig. 10). At mid-gastrulation, expression of *bmp2b*/*swr* is reduced in embryos that lack *vox* and *vent* function, and *vox* and *vent* are likewise reduced in *bmp2b*/*swr* mutants (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). These results show that zygotic BMP signals are required for normal levels of *vox* and *vent* expression during gastrulation. We suggest that *vox* and *vent* act to promote *bmp2b*/*swr* and *bmp4* expression by inhibiting the expression of *chd*, which is known from previous work to block a positive autoregulatory activity of BMP signals (Hammerschmidt et al., 1996b; Schulte-Merker et al., 1997). Accordingly, *vox* and *vent* do not activate *bmp* and other ventrally expressed genes directly, but instead repress *chordin*, an inhibitor of *bmp* expression (Fig. 10). In embryos that lack *vox* and *vent*, we propose that interruption of this *vox*/*vent*-*bmp2b* positive-feedback loop is responsible for the diminution of ventral gene expression at mid-gastrulation. The timing of changes in gene expression in embryos lacking *vox* and *vent* function is consistent with this model, because expression of *chd* is expanded significantly before the expression of *bmp* and other ventral genes is reduced. Interruption of the *vox*/*vent*-*bmp2b* positive-feedback loop may also lead to the expansion of some dorsal genes in *bmp2b*/*swr* mutants. Accordingly, *chd* expression is normal in *bmp2b*/*swr* mutants at the onset of gastrulation, but expanded by mid-gastrulation (Miller-Bertoglio et al., 1997), so that the expansion of *chd* coincides with the reduction of *vox* and *vent* expression in *bmp2b*/*swr* mutant embryos.

Our results suggest that the *vox*/*vent*-*bmp2b* positive feedback loop maintains ventral positional identity during gastrulation. The participation of the extracellular factors Chordin and BMP incorporates flexibility and sensitivity to cellular environment into the mechanism that maintains dorsoventral identity. For example, a cell moving from ventral to dorsal territories during gastrulation would reduce its expression of *vox* and *vent* in response to increased levels of Chd and reduced levels of BMP activity. The reduction of Vox and Vent levels would in turn permit the expression of dorsal genes appropriate for the cell's new environment.

bozozok is required to antagonize the action of *vox* and *vent*

The homeobox gene *boz* is required for the normal expression of *gsc*, a gene that is repressed by *vox* and *vent*. Our genetic evidence indicates that *boz* promotes dorsal mesodermal gene expression indirectly, by repressing *vox* and *vent* expression in the late blastula (Fig. 10). The *gsc* gene is expressed, and indeed greatly expanded, in *boz* embryos that also lack *vox* and *vent* function, indicating that the function of *boz* is not needed to promote *gsc* expression if *vox* and *vent* are inactive. Consistent with our evidence that a crucial function of *boz* is to repress the action of *vox* and *vent*, previous work indicates that the expression of *vox* and *vent* is expanded to include dorsal territories in *boz* mutants (Kawahara et al., 2000a; Kawahara et al., 2000b; Melby et al., 2000). In *boz* mutants, expression of *bmp2b*/*swr* is also expanded dorsally (Koos and Ho, 1999), and the evidence that *bmp2b*/*swr* and *vox*/*vent* are not required for each other's expression in the late blastula suggests that *boz* represses these genes in parallel (Fig. 10). The Boz protein has a motif that is conserved among transcriptional repressors (Koos and Ho, 1998), supporting the possibility that Boz may directly repress genes with ventralizing activity such as *vox*, *vent* and *bmp2b*. Conversely, our results show that *vox* and *vent* repress *boz* expression in a negative regulatory loop established in the late blastula (Fig. 10). Thus genetic analysis shows that the antagonistic transcription factors Vox/Vent and Boz are essential components of the mechanism that establishes and maintains dorsoventral asymmetry in the zebrafish.

Role of *vox* and *vent* in maintaining dorsoventral asymmetry

Characterization of embryos that lack *vox* and *vent* function demonstrates that the redundant action of these genes antagonizes expression of a number of dorsally expressed genes including *gsc*, *chd* and *boz*. Interestingly, we found that *chd*, *gsc*, and *boz* are expressed only on the dorsal side of Df^{st7} mutants at sphere stage (4 hours). Slightly later (dome stage, 4.3 hours), expression of *gsc* and *chd* is strongly expanded, and *boz* expression is expanded at 30% epiboly (4.7 hours). Thus it seems that the action of *vox* and *vent* is not required to repress dorsal genes at the time of their initial activation. Soon after initiation of dorsal gene expression, however, the redundant action of *vox* and *vent* is required to prevent expansion of dorsal genes into ventrolateral territories.

Why are *vox* and *vent* needed to repress dorsal gene expression in the late blastula but not in the initial phase of dorsal gene expression? We propose that an inducer of dorsal genes is present in the ventral marginal region of the late blastula, when and where *vox* and *vent* are required, but not at the midblastula period, when *chd* and *gsc* are dorsally restricted even in the absence of *vox* and *vent* function. β-catenin is a candidate for such an inducer. After its early role in development of dorsal axial structures (Heasman et al., 1994), β-catenin is required for development of ventral structures during gastrulation (Pelegri and Maischein, 1998; Heasman et al., 2000). In zebrafish, β-catenin begins to accumulate in ventrolateral marginal nuclei in the late blastula stage (Warga, 1996), approximately coinciding with the onset of the requirement for *vox* and *vent*. Thus *vox* and *vent* may prevent ectopic activation of dorsal gene expression by β-catenin acting in the ventral region of the late blastula. It is also likely that Vox and Vent counteract other activators of dorsal gene expression, including the Nodal-related signals Squint and Cyclops (Erter et al., 1998; Feldman et al., 1998; Rebagliati et al., 1998; Sampath et al., 1998). Like β-catenin, Nodal-related signals are potent inducers of *chd* and *gsc* expression in

overexpression assays. *squint* and *cyclops* are expressed around the circumference of the margin, where they induce mesendoderm formation, in the late blastula but not at earlier stages. Thus, Vox and Vent may be required to prevent Nodal-related signals from ectopically activating dorsal genes during mesendoderm induction. This model predicts that the Vox/Vent repressors counteract the effects of transcriptional mediators of Nodal signals and/or β-catenin, thereby restricting expression of genes such as *gsc* to the dorsal marginal region.

Conclusions

In summary, our results support the following model (Fig. 10) of the interactions among genes that establish dorsoventral asymmetry in zebrafish. The maternal dorsal determinant β-catenin activates expression of *boz*, which in turn represses *vox*, *vent*, and *bmp2b* in dorsal cells. *vox* and *vent* have redundant activities that repress genes characteristic of dorsal mesoderm, including *chd* and *gsc*. Zygotic BMP signals are not required for the repression of the dorsalmost mesodermal fates, but these signals are essential activators of ventral genes. Chordin antagonizes BMP signals, thereby inhibiting a positive feedback loop between *vox*/*vent* and *bmp2b* during gastrulation. Biochemical characterization of the target genes of Vox, Vent and Boz will elucidate the mechanisms by which the antagonistic activities of these genes establish the dorsoventral axis in the zebrafish embryo.

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