The molecular nature of zebrafish *swirl*: BMP2 function is essential during early dorsoventral patterning

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SUMMARY

Early dorsoventral pattern formation in vertebrate embryos is regulated by opposing activities of ventralizing bone morphogenetic proteins (BMPs) and dorsal-specific BMP antagonists such as Chordin, Noggin and Follistatin. Specific defects in early dorsoventral patterning have been recently found in a number of zebrafish mutants, which exhibit either a ventralized or dorsalized phenotype. One of these, the ventralized mutant *chordino* (originally called *dino*) is caused by a mutation in the zebrafish *chordin* homologue and interacts genetically with the dorsalized mutant *swirl*. In *swirl* mutant embryos, dorsal structures such as notochord and somites are expanded while ventral structures such as blood and nephros are missing. Here we demonstrate that the *swirl* phenotype is caused by mutations in the zebrafish *bmp2* gene (*zbmp2*). While injection of mRNAs encoded by the mutant alleles has no ventralizing effect, injection of wild-type *zbmp2* mRNA leads to a complete rescue of the *swirl* mutant phenotype. Fertile adult mutant fish were obtained, showing that development after gastrulation is not dependent on *zbmp2* function. In addition zBMP2 has no maternal role in mesoderm induction. Our analysis shows that *swirl/BMP2*, unlike mouse *BMP2* but like mouse *BMP4*, is required for early dorsoventral patterning of the zebrafish embryo.

Key words: zebrafish, mutation, *swirl*, BMP2, dorsoventral patterning

INTRODUCTION

The mechanisms of dorsal-ventral pattern formation in vertebrates are best understood in amphibians. In *Xenopus laevis*, during early cleavage stages, dorsal and ventral vegetal cells, respectively, induce dorsal and ventral mesoderm in neighboring cells of the marginal zone (reviewed by Slack, 1994). The molecular nature of the inducing signals is unknown, but members of the TGFβ family probably play a crucial role. Subsequently, prior to and during gastrulation, various factors such as BMPs and Wnts on the ventral side and their antagonists within the dorsal organizer specify the different ventral and dorsal fates of the mesodermal cells, such as blood, nephros, muscle and notochord (Moon et al., 1997).

As far as ventral signals are concerned, Bone morphogenetic protein-4 (BMP4) has received most attention as a candidate for ventralizing activity, while various other members of the BMP family have been either implicated or shown to play a role in numerous embryological processes, based on their expression patterns and on the result of gene targeting experiments (reviewed by Hogan, 1996, and see below). BMP4 has been recognized as a potent ventralizing factor (Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Neave et al., 1997; Nikaido et al., 1997) in both frog and fish embryos, where RNA overexpression in embryos leads to a strong reduction of dorsal structures. In frogs, *bmp4* is expressed in cells of the animal pole

and in ventrolateral regions of the marginal zone but is excluded from the organizer region (Fainsod et al., 1994). In zebrafish, the situation is different in that *bmp4* is also expressed at low levels in the embryonic shield, while *bmp2* is confined to ventrolateral regions (Nikaido et al., 1997). Mesoderm dorsalization occurs when BMP4 signaling is inhibited by a dominant negative BMP receptor (Graff et al., 1994; Suzuki et al., 1994), dominant negative ligand (Hawley et al., 1995) and antisense *bmp4* RNA (Steinbeisser et al., 1995), suggesting that a functional BMP4 signaling pathway is essential for ventral specification in the *Xenopus* embryo.

Biochemical studies show that *chordin* (Sasai et al., 1994), *noggin* (Smith and Harland, 1992), and *follistatin* (Hemmati-Brivanlou et al., 1994), all of which are secreted polypeptides expressed by an embryonic structure called the organizer, can directly bind to BMP4, and block binding of BMP4 to its natural receptor (Piccolo et al., 1996; Zimmerman et al., 1996; Fainsod et al., 1997). The antagonistic interaction between BMP2/4 and Chordin probably establishes an activity gradient of BMP, and the gradient plays an important role in dorsal-ventral pattern formation in the *Xenopus* embryo. This patterning system is functionally conserved in the *Drosophila* embryo (Padgett et al., 1993; Holley et al., 1995). Genetic studies revealed that *short-gastrulation* (*sog*) acts as an antagonist of *decapentaplegic* (*dpp*), and the resulting *dpp* activity gradient organizes dorsal-ventral patterning (Ferguson and

Anderson, 1992; Wharton et al., 1993). *sog* is the homologue of *Xenopus chordin* (Sasai et al., 1994) and the zebrafish gene *chordino* (Schulte-Merker et al., 1997), which is expressed in dorsal structures such as embryonic shield and neuroectoderm in the zebrafish gastrula (Schulte-Merker et al., 1997; Schulte-Merker and Hammerschmidt, unpublished).

Recently, it was shown that the *chordino* mutant (previously named *dino*; Hammerschmidt et al., 1996b) is a mutant in the *chordino* gene (Schulte-Merker et al., 1997). The *chordino* mutant has a strongly ventralized phenotype, and *chordino* and *swirl* have antagonizing functions in the zebrafish embryo (Hammerschmidt et al., 1996a).

Consistent with the notion of a Dpp/BMP activity gradient, BMP4 mutant mouse embryos have severe abnormalities in embryonic mesodermal structures (Winnier et al., 1995). Most of them die at the egg cylinder stage when mesodermal formation normally commences. Some homozygous embryos that develop further show retarded development, and have deficiencies of hematopoietic cells and posterior structures. This demonstrates that in mouse development, consistent with over-expression studies in *Xenopus* and fish, BMP4 plays an important role in development of ventroposterior mesoderm. In contrast, *bmp2*, which is expressed in extraembryonic mesoderm and the precardium of mouse embryos (Lyons et al., 1989,1990 and 1995), has a role in amnion and heart development, as demonstrated by the phenotype of homozygous mutant embryos (Zhang and Bradley, 1996).

In recent years, large scale mutant screens were performed in the zebrafish, *Danio rerio*, yielding mutants in various developmental processes (Haffter, 1996; Driever et al., 1996). Among them, several mutations which cause dorsal-ventral patterning defects were isolated (Mullins et al., 1996; Hammerschmidt, 1996b; Solnica-Krezel et al., 1996). Mutants in at least 6 genes (pending complementation tests between mutants from the two screens) show different strengths of dorsalized phenotypes (Mullins et al., 1996). Embryos mutant in *swirl* (*swr^{ta72}* and *swr^{tc300}*) exhibit the most severe degree of dorsalization and completely lack ventral structures such as blood and pronephros, as judged by morphology and altered expression patterns of marker genes. In *swirl* embryos, the region expressing dorsal and dorsolateral markers such as *no tail*, *fkd 3* and *myoD* is expanded, whereas expression of ventral markers such as *eve1* and *gata1* are reduced (Mullins et al., 1996; this paper). The phenotype of *swirl* mutant embryos resembles that of embryos injected with mRNA encoding a dominant negative BMP receptor (Hammerschmidt et al., 1996a). This suggests that the BMP signaling pathway may be interrupted in *swirl* mutant embryos.

Here we demonstrate that *swirl* encodes the zebrafish homolog of *bmp2*. The dorsalized *swirl* mutant phenotype can be completely rescued by injection of *zbmp2* mRNA, which allows us to raise mutant fish to adulthood and to demonstrate that BMP2 plays no maternal role in mesoderm induction. Our findings indicate that *zbmp2* functions during dorsoventral patterning in a role analogous to that of *bmp4* in mouse embryogenesis.

MATERIALS AND METHODS

Fish

Zebrafish stocks were maintained as described previously (Haffter,

1996). All experiments were performed using the Tübingen wild-type strain, and *swr^{ta72}* and *swr^{tc300}* mutant alleles (Mullins et al., 1996). Embryos were obtained by natural matings.

Cell transplantations

Cell transplantation experiments were performed essentially as described by Ho and Kane (1990). Donor embryos were dye-labeled by injection of either a mixture of tetramethylrhodamine dextran and lysine fixable biotinylated dextran, or lysine fixable fluorescein dextran (Molecular Probes). All dyes were disolved at a final concentration of 5% (w/v) in 0.2 M KCl. Host embryos were not labeled. All embryos were dechorionated on 1.5% agarose plates prior to transplantation. At sphere stage, 5-10 cells were taken from one labeled donor embryo using a transplantation pipette, and then another 5-10 cells from a differently labeled donor embryo were drawn up into the same pipette. Donor cells were transplanted simultaneously into unlabeled host embryo. To record the positions of the transplanted cells in respect to the embryonic shield, donor cells were visualized at the shield stage using an Axioskop microscope (Zeiss) equipped with epifluorescence optics and a CCD camera with image intensifier (Videoscope), and images were recorded using AxioVideo image softwear. Host embryos were fixed at 36 hours in 4% paraformaldehyde and processed using the ABC kit (Vector Laboratories) for detection of biotinylated dextran-containing cells, and anti-fluorescein antibody-alkaline phosphatase conjugate (Boehringer) for fluorescein dextran-containing cells.

RNA injection

Capped mRNAs were synthesized using SP6 RNA polymerase from linearized pSP64T-based constructs as described previously (Hammerschmidt et al., 1996a) and were injected at the 1-2 cell stage in 80 pl of 0.2 M KCl using an Eppendorf Microinjector 5242.

Artificial mutant *zbmp2* mRNA was prepared as follows. The pSP64T-plasmid containing the *zbmp2* cDNA was linearized with *Apa*I, blunt-ended and self-ligated with T4 DNA ligase. The construct produces *zbmp2* mRNA containing 4 additional bases in the pro-domain cording region, which results in a frameshift.

Molecular methods

Total RNA was prepared as previously described (Schulte-Merker et al., 1992). First strand cDNA synthesis was carried out using Superscript (GIBCO BRL) according to the manufacturer's instructions. Genomic DNA of adult fish was prepared as described by Schulte-Merker et al. (1997). PCR reactions were carried out using either first strand cDNA or genomic DNA as a template. The enzyme used was Pfu DNA polymerase (Stratagene). The following primers were used: for the whole coding region of *zbmp2*: sense primer: 5′ GAA-GATCTCTCCTCCGGAACTGACTG 3′, antisense primer: 5′ GAA-GATCTGTTCATTCAACATAAATATATTC 3′; for the mature protein coding region of *zbmp2*: sense primer: 5′ GCAAAGCCGAGGA-GAAAGC 3′, antisense primer: 5′ TCCTCCAAAATAGCTCGCTC 3′. The PCR product of the fragment containing the whole coding region of *zbmp2* was digested with *Bgl*II, purified by agarose gel electrophoresis and cloned into the pSP64T vector (Krieg and Melton, 1984). All other PCR fragments were cloned using the TA cloning kit (Invitrogen).

Sequencing was performed on an ALF sequencer (Pharmacia). In all cases, both strands were sequenced.

Sequencing of alleles

At the time of this analysis we had no heterozygous females available for *swr^{tc300}*. By crossing *swr^{tc300}* heterozygous males with *swr^{ta72}* heterozygous females, we obtained transheterozygous mutant *swirl* embryos. RT-PCR fragments containing the open reading frame of *zbmp2* were amplified and cloned and 6 independent clones were sequenced. Four of them showed the sequence alteration as previously found in *swr^{ta72}*. The other two clones showed a base substitution

(Fig. 3A) which replaces a cysteine in the mature domain of zBMP-2 with a tryptophan (TGT→TGG).

To confirm this finding, we used genomic DNA from an adult *swr*[tc300] heterozygous fish as a template to generate a PCR product containing the site in which the base substitution was found. Sequences of the clones analysed were either wild-type (in 4 out of 6 cases) or showed the sequence alteration in the third position of the cysteine residue (in 2 out of 6 cases).

Single-strand conformation polymorphism (SSCP) analysis

Genomic DNA was extracted from single embryos using the method described previously (Westerfield, 1993). PCR amplifications were performed using Taq DNA polymerase (Pharmacia) with 500 ng of genomic DNA as templates in 20 μl reaction. The primers used were: TCCAACATTCCCAAAGCCTG as the sense primer, and TCCTC-CAAAATAGCTCGCTC as the antisense primer. SSCP analysis was performed as described previously (Orita et al., 1989). The PCR products were precipitated with 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate, and redissolved in 12 μl of water. 5.4 μl of the respective samples were mixed with 0.6 μl of denaturing solution (10 mM EDTA, 500 mM NaOH) and 2.4 μl of loading buffer (consisting of 1 ml formamide, 1 μl saturated bromophenol blue, and 1 μl xylenecyanol). The mixtures were incubated for 10 minutes at 80°C and chilled on ice. 7 μl of each sample were loaded on native precast acrylamide gels (CleanGel DNA-HP 15%, ETC Elektrophorese-Technik) supported by Multiphor II Flatbed Electrophorasis system (Pharmacia), and electrophoresed at even temperature (15.0°C). The gels were processed using a silver staining kit (Pharmacia).

In situ hybridization

Whole-mount in situ hybridization was performed as previously described (Schulte-Merker et al., 1994; Hammerschmidt et al., 1996a).

RESULTS

Xenopus BMP4 rescues the *swirl* mutant phenotype

BMP4, a member of the TGFβ superfamily, has been demonstrated in a number of assays to be able to ventralize mesoderm (Dale et al., 1992; Jones et al., 1992; Neave et al., 1997; Nikaido et al., 1997), and it has been shown previously that *swirl* mutant embryos are partially rescued by injection of *xbmp4* DNA under the control of the cytoskeletal actin promoter (Hammerschmidt et al., 1996a). We tested whether *swirl* mutant embryos can be rescued completely by microinjecting *xbmp4* mRNA, and whether the dorsalized phenotype can be reverted into a ventralized one. The result is shown in Table 1. Homozygous uninjected embryos showed a strongly dorsalized phenotype (C5), with all posterior and ventral structures missing (Fig. 1A). In embryos injected with more than 2 pg of mRNA, strongly dorsalized embryos were completely missing as a consequence of the ventralizing activity of XBMP4, and less severely dorsalized phenotypes were found (Fig. 1B-E). At the same time, the number of normal embryos (Fig. 1F) decreased and the fraction of ventralized embyros (Fig. 1G-J) was increased. This suggests that the weaker dorsalized embryos (C1-4) are likely to be homozygous mutant embryos rescued by mRNA injection. In embryos injected with 3 pg of mRNA or more, the proportion of dorsalized embryos totalled 1%, indicating that normal and weakly ventralized embryos must contain rescued mutant embryos.

Table 1. The effects of XBMP-4 mRNA injection into *swr*[ta72] embryos

Amount of mRNA injected (pg)	Dorsalized (%)					Normal (%)	Ventralized (%)				*n*
	C5	C4	C3	C2	C1		V1	V2	V3	V4	
0	27	0	0	0	0	73	0	0	0	0	62
0.4	15	0	0	0	0	85	0	0	0	0	32
1.0	35	0	3	0	0	62	0	0	0	0	31
2.0	0	5	0	13	3	56	10	5	3	3	39
3.0	0	0	0	0	3	52	12	18	12	3	33
4.0	0	0	0	0	0	6	3	31	40	20	35
40.0	0	0	0	0	0	3	0	0	0	97	29

swirl acts non cell-autonomously

To determine whether *swirl* acts in a cell autonomous fashion, we carried out cell transplantation experiments. Lineage-tracer labelled mutant cells were transplanted into wild-type embryos. The position of the transplanted cells was monitored at shield stage, and embryos were scored at tailbud stage and at 24 hours postfertilization. Donor embryos were labeled either with a mixture of rhodamindextran (RDA) and biotin-dextran (BDA), or with fluoresceindextran (FDA). Small groups of 10-20 cells from two distinctively labeled donor embryos were drawn up into the same transplantation pipette and cotransplanted into unlabeled embryos from two adults heterozygous for *swirl*. Donor embryos were produced by crossing double heterozygotes for *swirl* and *dino*, and kept to develop to determine their phenotype. We compared fates and locations of *swirl*, *dino* and wild-type cells.

Homozygous *swirl* embryos lack ventral structures such as blood and pronephros. Surprisingly, when *swirl* mutant cells were transplanted into the ventral side of wild-type embryos (Fig. 2A, red: swirl, green: wild-type), in some cases they were observed to differentiate into blood cells (Fig. 2A, brown cells). Wild-type cells, cotransplanted with *swirl* cells, also gave rise to blood cells (Fig. 2A, blue cells). Before fixation of the embryo, we observed red and green fluorescent cells circulating through blood vessels in the same live embryos (data not shown). When *swirl* cells were cotransplanted together with *dino* cells into the ventral side of host embryos (Fig. 2B), both cell populations gave rise to blood (Fig. 2B). As expected, *swirl* mutant cells also gave rise to various other cells types, such as muscle (Fig. 2C) and notochord (Fig. 2D). We did not observe significant differences between the fates and locations of *swirl*, *dino* and wild-type cells in host wild-type embryos when two groups of cells were transplanted at the same position. These results demonstrate that *swirl* acts non cell-autonomously.

Mutations in *swr*[ta72] and *swr*[tc300]

As *swirl* acts non cell-autonomously, and as XBMP4 can rescue the *swirl* mutant phenotype, we expected the *swirl* gene product to encode a secreted molecule involved in BMP signaling. In zebrafish, two BMP-related genes, designated as *zbmp2* and *zbmp4*, have been reported (Nikaido et al., 1997; Lee and Zon, unpublished). In order to determine whether *swirl* encodes BMP2 or BMP4, we prepared poly(A)+ RNA from mutant *swirl* embryos at the 2 somite stage, and obtained RT-PCR fragments that spanned the translated region of both *zbmp2* and *zbmp4*. No difference was found between the deduced amino acid sequence of zBMP4 from *swr*[ta72] and
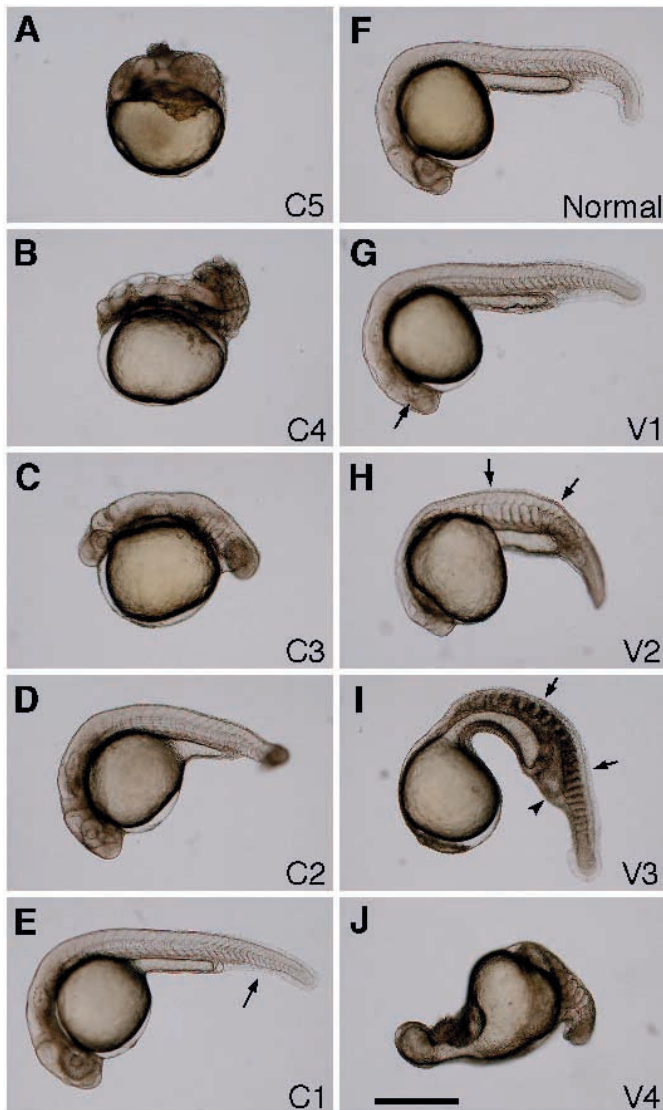
Fig. 1. Phenotypes of embryos from two heterozygous *swr^{ta72}* parents injected with *xbmp4* mRNA. (A) Uninjected *swr^{ta72}* homozygous embryo showing the dorsalized C5 phenotype. (B-J) Injected embryos. (B) Dorsalized C4. (C) Dorsalized C3. (D) Dorsalized C2. (E) Dorsalized C1 phenotype. Only the ventral tail fin (arrow) is affected, and most of these embryos survive. (F) Normal embryo. (G) Ventralized V1 embryo with anterior neural defects (arrow points to reduced eyes), but with a notochord. (H) Ventralized V2 embryo with reduced or absent notochord, expanded posterior somites (arrows), but remaining head structures. (I) Ventralized V3 embryo with little or no head structures left, no notochord, and expanded posterior somites (arrows) as well as enlarged blood islands (arrowhead). (J) Ventralized V4 embryo which is grossly abnormal, lacks all anterior structures, but still shows somites. Scale bar, 500 μm.

wild-type (data not shown). However, in the case of zBMP2, a base substitution was found in the termination codon of *swr^{ta72}* (Fig. 3A), altering the termination codon into a tryptophan codon (TGA→TGG). Consequently, the resulting conceptual protein uses the next in frame stop codon, which follows after 18 nucleotides. The resulting zBMP2 mutant protein therefore contains an additional 6 amino acids at the carboxy end of.

In the case of the second *swirl* allele, *swr^{tc300}*, we found a base substitution (Fig. 3A) which exchanges a cysteine in the mature domain of zBMP2 into a tryptophan (TGT→TGG). Additionally, we sequenced PCR fragments of *zbmp2* amplified from genomic DNA of the relevant founder fish which was used for the initial mutagenesis in the Tübingen mutant screen. As expected, we found no base substitution in the relevant fragment from the founder fish, confirming that the base substitution had been induced by the ENU-mutagenesis.

swirl and *zbmp2* are linked

To investigate genetic linkage between *swirl* and *zbmp2*, single-strand conformation polymorphism (SSCP) analysis was performed (Orita et al., 1989). In preliminary experiments, a 189 base-pair fragment containing the stop codon was amplified from both a mutant *swr^{ta72}* cDNA and wild-type genomic DNA. When the PCR products were analysed by SSCP, the respective products yielded different SSCP patterns (data not shown).

The ability to distinguish wild-type and mutant forms of *zbmp2* allowed us to test linkage between *zbmp2* and *swr^{ta72}*. Genomic DNA was extracted from 38 individual mutant and 38 individual wild-type sibling embryos, and analysed by SSCP. Fig. 3B shows representative SSCP patterns of PCR products from 4 mutants (lane 1-4) and 4 wild-type siblings (lane 5-8). Three distinctive patterns were observed. Pattern A was observed in all 38 mutants as shown in lane 1-4. This pattern is identical to the pattern from the *swr^{ta72}* cDNA (not shown, but compare Fig. 4, lane 1). The second type of pattern was observed in siblings (pattern B: lanes 5 and 8) and in the case of a wild-type *zbmp2* cDNA (not shown, but compare Fig. 4, lane 10). The third pattern C (lanes 6 and 7) consists of an addition of the A and B pattern, suggesting that pattern B represents the wild-type *swirl* (+/+) situation and that pattern C stems from heterozygous embryos. This interpretation is consistent with the fact that pattern B was observed in 9 siblings, whereas pattern C was seen in 29 siblings. This demonstrates that *swirl* and *zbmp2* are closely linked genetically (not more than 1.3 cM apart).

swirl mutant embryos can be rescued by wild-type *zbmp2* mRNA, but not by mutant mRNAs

In order to investigate whether *zbmp2* mRNA can rescue the *swirl* mutant phenotype, synthetic mRNA of wild-type *zbmp2* was injected into embryos produced by crossing heterozygous fish for *swr^{ta72}*. As expected, the proportion of C5 dorsalized embryos was significantly reduced (Table 2), and it is worth noticing that the amount of RNA needed to achieve rescue was lower than when *xbmp4* was used (compare with results from Table 1). To confirm that some of the embryos which showed a wild-type phenotype were homozygous for *swirl*, we genotyped eight of these embryos by SSCP analysis. Genomic DNA was extracted from single embryos and analysed by SSCP as described above (see Fig. 3A). In 2 out of 8 embryos, the SSCP pattern indicative of *swr^{ta72}* homozygotes was observed. Fig. 4A and B show a representative example of a rescued *swirl* homozygote and of a wild-type sibling. This demonstrates that homozygous embryos for *swirl* can be rescued completely by *zbmp2* mRNA injection.

To test to what extent the activity of zBMP2 is reduced by the base substitutions in each *swirl* allele, mRNA generated from *swr^{ta72}* and *swr^{tc300}* constructs was injected into *swirl*

Fig. 2. Cell transplantation analysis of *swirl* and *dino*. Mutant or wild-type donor cells were transplanted into wild-type host embryos. The genotype of the donor cells is indicated at the lower left of each picture. All embryos were fixed and stained at 36 hours. Brown or blue cells correspond to cells containing mixture of rhodamine dextran and biotin dextran, or fluorescein dextran, respectively. Animal pole views of embryos at shield stage are shown in insets with dorsal to the right. (A) *swirl* mutant cells (brown) can differentiate into blood cells (arrow), as do cotransplanted wild-type cells. (B) *dino* (brown) and *swirl* (blue) mutant cells form blood in a wild-type environment. (C) *swirl* (brown) and *dino* (blue) mutant cells can differentiate into muscle cells. (D) *swirl* (brown) and wild-type (blue) can form notochord cells. Scale bar, 50 µm.
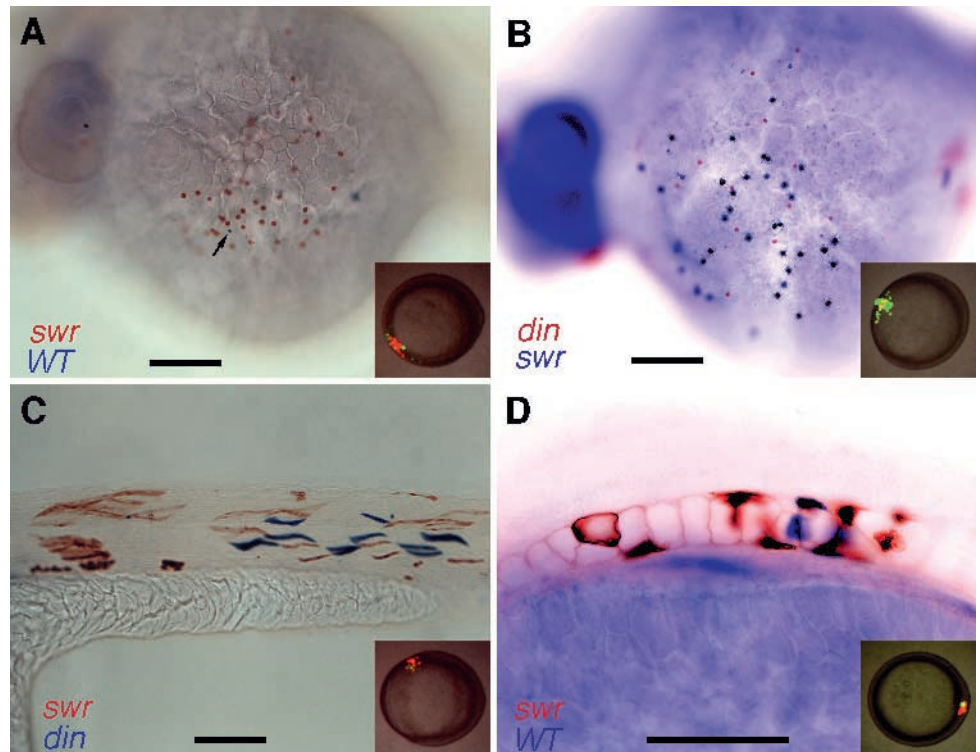
embryos. In contrast to injections using wild-type *zbmp2*, ventralized embryos were never observed, and the proportion of C5 dorsalized embryos was not decreased, even when embryos were injected with up to 20 pg of the respective mutant cDNAs (Table 2). These experiments clearly demonstrate that the activities of zBMP2 from mutant alleles are strongly reduced.

swr^ta72 mRNA exhibits dorsalizing activity

Interestingly, in embryos injected with 4 pg of *swr^ta72* mRNA, the proportion of normal embryos was strongly reduced, while some weakly dorsalized embryos were observed. This suggests that the mutant protein might exhibit a dominant negative function, for example by interfering with other BMPs in the embryo. To test this we injected wild-type and mutant *zbmp2* mRNAs into wild-type embryos. In embryos injected with 4 pg of wild-type *zbmp2* mRNA, all embryos were ventralized, and 75% of the injected embryos showed the severest possible phenotype (Table 3). In contrast, 4 pg of *swr^ta72* mRNA did not result in ventralization of embryos, but most embryos showed a weak C1 or C2 dorsalized phenotype. The most severe phenotypes observed were C3 phenotypes (Table 3). *swr^tc300* mRNA caused neither ventralization nor dorsalization of wild-type embryos when 4 pg were injected, but injection of even higher amounts causes strong dorsalization (Table 3). Injection of a *zbmp2* control mRNA carrying a frame shift mutation (see Materials and Methods) did not affect the phenotype of embryos. Therefore these mutant proteins behave as dominant-negative products.

swirl mutants lack blood and heart precursors

Mouse embryos lacking a functional copy of *bmp2* show deficiencies in blood and heart formation (Zhang and Bradley, 1996). We have analysed the appearance of both these tissues in *swirl* mutant embryos by carrying out in situ hybridizations for *gata1*

and *Nkx2.5/Nkx2.7*. *gata1* is expressed in blood precursors at the 8 somite stage (Detrich et al., 1995), while *Nkx2.5* and *2.7* are expressed in cardiac mesodermal precursors (Lee et al., 1996).
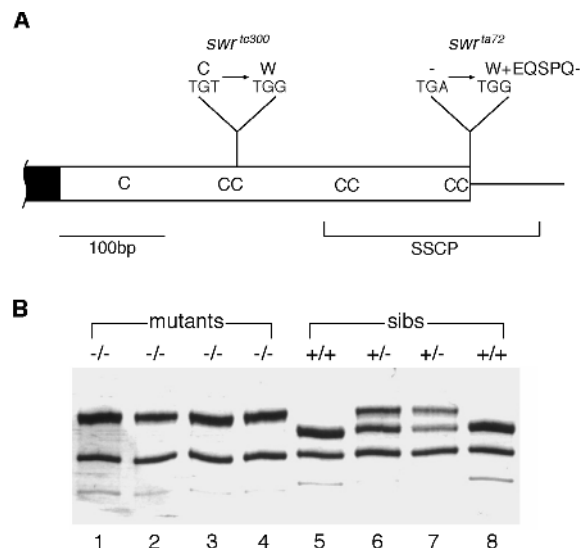

Fig. 3. Molecular lesions in the *zbmp2* open reading frame of *swirl*. (A) Schematic representation of mutations of *swr^ta72* and *swr^tc300* alleles in the *zbmp2* gene. The biologically active, mature region of zBMP2 (white box) contains seven characteristic cysteine residues, which are almost invariantly conserved in members of the TGFβ superfamily. In *swr^tc300*, a cysteine is substituted by a tryptophan residue. In *swr^ta72*, the stop codon is also changed into a tryptophan residue, leading to the addition of an extra 6 amino acids. The region used for SSCP analysis is indicated. (B) SSCP linkage analysis to demonstrate linkage between *swirl* and *zbmp2*. Representative patterns of *swr^ta72* mutants (lanes 1-4) and sibling embryos (lanes 5-8) are shown.

Table 2. Comparison of the effects of wild-type and mutant zBMP-2 mRNA injection on *swr^ta72* embryos

Type of mRNA injected	Amount of mRNA injected (pg)	Dorsalized (%)					Normal (%)	Ventralized (%)				n
		C5	C4	C3	C2	C1		V1	V2	V3	V4	
Uninjected	0	32	0	0	0	0	68	0	0	0	0	148
Wild-type zBMP-2	1	0	0	4	2	0	37	26	7	14	11	57
	2	0	0	0	0	0	0	8	12	35	45	65
	4	0	0	0	0	0	1	14	17	20	47	70
swr^ta72 zBMP-2	1	23	0	0	0	0	77	0	0	0	0	30
	2	38	0	0	6	9	47	0	0	0	0	34
	4	32	6	12	14	18	18	0	0	0	0	34
swr^tc300 zBMP-2	1	27	2	0	0	6	65	0	0	0	0	52
	2	22	0	0	0	0	78	0	0	0	0	50
	4	23	3	0	3	7	63	0	0	0	0	30
	20	58	17	8	4	4	8	0	0	0	0	24
Artificial Mutant zBMP-2	4	32	0	0	0	0	68	0	0	0	0	28
	20	19	0	0	0	0	81	0	0	0	0	36

The absence of *gata1*-positive blood precursor cells in *swirl* mutants has been previously reported (Mullins et al., 1996), and was confirmed by us (data not shown). The effects of the *swirl* mutant phenotype on heart morphogenesis had not been addressed previously. Using both *Nkx2.5* and *Nkx2.7* (Lee et al., 1996) as markers for cardiac mesodermal precursors, we found that expression of *Nkx2.5* is completely absent in the majority of homozygous mutant embryos, while in wild-type embryos two bilateral rows of cells express *Nkx2.5* (Fig. 5A,B). In a minority of mutant embryos, we found a few patches of cells positive for *Nkx2.5* at ectopic positions (Fig. 5C). The results obtained for *Nkx2.7* are essentially the same (data not shown).

Generation of *swirl* homozygous adult fish

We wished to investigate whether *swirl* homozygous embryos can give rise to adult fish. 215 embryos produced by *swr^ta72* heterozygotes were each injected with 3 pg of *xbmp4* mRNA. 79 embryos did not survive day 1. However, 86 embryos showed a normal phenotype, 7 were weakly dorsalized and 43 weakly ventralized. Of those, 111 fish reached adulthood. Genotyping was performed for the normal-looking fish both by SSCP analysis, using genomic DNA extracted from tail fins, and by crossing against *swirl* heterozygotes. So far, we have

identified two female and two male *swirl* homozygotes from 30 fish tested. They produced a total of 52% C5 mutant (*n*=464) and 48% normal (*n*=425) offspring when crossed against *swirl* heterozygotes.

Adult mutant fish of both sexes were fertile and appeared phenotypically normal. It is clear, therefore, that zBMP2 is not required for viability and fertility.

swirl does not have a maternal effect in mesoderm induction

Both BMP2 and BMP4 have been implied to induce ventral mesoderm in vivo. The progeny of mutant females appeared completely normal when crossed to a wild-type male, as assayed by observing live embryos microscopically, and by performing in situ analysis using *ntl* (Schulte-Merker et al., 1992), *gata1* (Detrich et al., 1995), and *myoD* (Weinberg et al., 1996) as markers for normal dorsoventral polarity (data not shown). Therefore, *swirl* does not have a maternal effect in mesoderm induction.

Expression of *zbmp2* and *zbmp4* is affected in *swirl* mutants

To gain information about the regulation of *zbmp2* expression and the interplay of zBMP2 and zBMP4, we studied the

Table 3. Comparison of the effects of wild-type and mutant zBMP-2 mRNA injection on wild-type embryos

Type of mRNA injected	Amount of mRNA injected (pg)	Dorsalized (%)					Normal (%)	Ventralized (%)				n
		C5	C4	C3	C2	C1		V1	V2	V3	V4	
Uninjected	0	0	0	0	0	0	100	0	0	0	0	50
Wild-type zBMP-2	2	0	0	0	0	0	19	26	7	14	11	21
	4	0	0	0	0	0	0	8	12	35	45	20
swr^ta72 zBMP-2	2	0	0	0	4	0	96	0	0	0	0	24
	4	0	0	5	24	48	24	0	0	0	0	21
swr^tc300 zBMP-2	2	0	0	0	0	0	100	0	0	0	0	19
	4	0	0	0	0	0	100	0	0	0	0	19
	20	84	12	0	0	0	4	0	0	0	0	25
Artificial Mutant zBMP-2	20	0	0	0	0	0	100	0	0	0	0	23
	40	0	0	0	0	0	100	0	0	0	0	26

expression patterns of both *zbmp2* and *zbmp4* in wild-type and *swirl* mutant embryos. In wild-type embryos, both *zbmp2* and *zbmp4* initially display a rather broad ventrolateral expression domain which extends far into the dorsal side of the pregastrula and becomes progressively restricted to ventral regions during the late blastula and gastrula stages (Nikaido et al., 1997). However, striking differences between *zbmp2* and *zbmp4* expression were found in the degree of this ventral restriction: while the expression of *zbmp2* is maintained in both marginal and animal ventral regions and eventually is present in ventral and lateral regions surrounding the entire body axis (Fig. 6E,I,J), the expression of *zbmp4* becomes restricted to the ventral marginal zone and is later found in posterior regions of the postgastrula embryo (Fig. 6G,K). In addition to the ventral expression, both *zbmp2* and *zbmp4* are expressed in a subset of cells on the dorsal side of the zebrafish embryo. This expression starts before the onset of gastrulation in dorsal marginal cells of the presumptive organizer (Fig. 6A, arrowhead). The *zbmp4* expression domain moves anteriorly during the course of gastrulation (Fig. 6G,K, arrowhead), indicating *zbmp4* expression is maintained in a subset of organizer cells that eventually form the hatching gland precursors. In contrast, the expression domain of *zbmp2* remains in the marginal region throughout gastrulation (Fig. 6A,E, arrowhead). Thus, *zbmp2* expression in dorsal cells is rather transient and only found while cells involute.

The dorsal expression of both *zbmp2* and *zbmp4* is independent of zBMP2 and normal in *swirl* mutant embryos (Figs. 6B,F,H, arrowhead). However, *zbmp2* and *zbmp4* are affected to different degrees in ventral regions of *swirl* mutant embryos (Figs. 6B,D,F,H). *zbmp2*, whose expression starts at midblastula stages (Nakaido et al., 1997), shows an indistinguishable expression pattern in *swirl* mutant embryos and their wildtype siblings at 30% and 40% epiboly (not shown). At the onset of gastrulation, *zbmp2* transcripts disappear very rapidly in animal ventral regions of *swirl* mutant embryos, but remain to be found at almost normal levels in the ventral marginal zone and the Yolk Syncytial layer (Fig. 6E,F). In contrast, the marginal expression of *zbmp4*, which starts at late blastula stages significantly later then *zbmp2* (Nikaido et al., 1997), comes up at strongly reduced levels (Fig. 6C,D; 50% epiboly), and disappears completely at early gastrula stages (60% epiboly, see Fig. 6H for 80% epiboly). This indicates that both initiation and maintenance of the ventral *zbmp4* expression depend on zBMP2. In addition, the maintenance of the animal expression of *zbmp2* itself requires functional zBMP2 protein, while the marginal *zbmp2* expression is maintained independently of zBMP2.

DISCUSSION

In this study, we show that BMP2 plays an essential role during the establishment of the dorsoventral axis in zebrafish embryos. Therefore, BMP2 function in fish seems to be more similar to the function of mouse BMP4 than to mouse BMP2.

swirl encodes *zbmp2*

We have demonstrated that *swirl*, a gene which if mutated leads to a strong dorsalization of the zebrafish embryo, encodes zBMP2, a member of the BMP-related proteins in zebrafish. First, in each of the alleles base substitutions in the open reading frame of *zbmp2* were found. Second, these base substitutions (Fig. 3A) lead to a complete loss of ventralizing activity in injection assays (Tables 2 and 3). Third, *swirl* and *zbmp2* are closely linked genetically (Fig. 3B). In 38 diploid embryos, no recombination was detected between *swirl* and the *zbmp2* locus, showing that *swirl* and *zbmp2* are at most 1.3 cM apart. Fourth, *swirl* mutant embryos were rescued by microinjection with wild-type *zbmp2* mRNA but not with mutant mRNA (Table 2), suggesting that the base substitutions disturb native zBMP2 activity. Thus, our findings show that ventral cell fate specification in the zebrafish embryo is mediated via zBMP2.

swr[tc300] and *swr*[ta72] zBMP2 completely lack ventralizing activity but might act as dominant negative dorsalizing factor

BMPs are members of the transforming growth factor-β (TGFβ) superfamily. They are initially synthesized as pre-pro-proteins, with a signal sequence and a pro-domain of varying size at the N terminus (Kingsley, 1994). The precursor protein is cleaved to yield the biologically active, mature carboxy-terminal protein, which then dimerizes. Almost all TGFβ molecules contain a nearly invariant array of 7 cysteine residues within the mature region of the protein. Crystallography studies of TGF-β2 suggest that the cysteine residue at position 4 links two monomers into a dimer, while the other six cysteines form a so-called cysteine knot (Daopin et al., 1992; Schlunegger and Grutter, 1992) in each monomer.

Mutation in *swr*[tc300] causes exchange of one of 7 cysteines in the mature domain into a tryptophan (Fig. 3A) which most likely causes the disruption of the cysteine knot, resulting in conformational changes in the zBMP2 monomer.

In the other allele, *swr*[ta72] allele, a base substitution in the stop codon of *zbmp2* results in the addition of 6 amino acids at the carboxy terminus of the conceptual zBMP2 protein (Fig. 3A). It is possible that the elongated amino acid tail interferes with the binding of zBMP2 to its receptor. While it is unclear at present what the effect of the elongated protein is, it is worth noticing that all BMPs and the large majority of all TGFβ molecules show the amino acid sequence CXCX at their C-terminal end. Thus, this region of the protein is highly conserved and our analysis demonstrates that the function of zBMP2 is impaired by the addition of 6 amino acids at the C terminus.

Interestingly, injection of both *swr*[ta72] and *swr*[tc300] mRNAs lead to dorsalization of wild-type embryos (Table 3). This result suggests the possibility that *swr*[ta72] and *swr*[tc300] may function as dominant negative forms capable of interfering with either the endogenous wild-type zBMP2 protein, or/and with other BMPs. Since mutant zBMP2 protein also exhibits a dominant negative effect in *swirl* mutant embryos and therefore in the absence of wild-type BMP2 protein, we suggest that the protein products of *swr*[ta72] and *swr*[tc300] either interfer with the receptor or heterodimerize with other BMPs. It is worthwhile to note that *swr*[ta72] initially displayed a weak dominant zygotic dorsalized phenotype before the fish had undergone several rounds of outcrosses (Mullins et al., 1996). Our molecular analysis of *swr*[ta72] offers an explanation for this observation.

Phenotypic comparison between *swirl* mutants and BMP knock-out mice
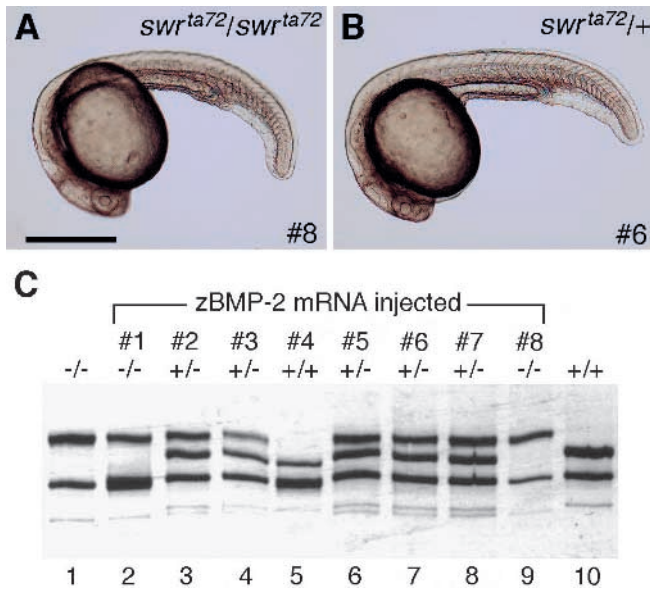
There are considerable phenotypical differences between

Fig. 4. Rescue of *swr^ta72* homozygous embryos by *zbmp2* mRNA injection. (A) A representative example of a *swr^ta72* homozygous embryo completely rescued by *zbmp2* mRNA injection. (B) A heterozygous sibling embryo. (C) SSCP genotyping of *zbmp2* mRNA injected embryos which show a normal phenotype. Lane 1: *swr^ta72* mutant, uninjected. Lanes 2-9: mRNA-injected embryos (#1-8). Lane 10: wild-type homozygote. Embryo #8 yields the same pattern as the *swr^ta72* homozygous embryo (lane 1). Embryo #8 is shown in A; embryo #6 is shown in B. Scale bar in A, 500 μm.

mouse and zebrafish *bmp2* mutants. By gene targeting technology using embryonic stem cells, mice deficient for the BMP2 mature region were produced (Zhang and Bradley, 1996). Homozygous embryos die around 9.5 days post coitum with defects in development of the amnion/chorion and the heart. In fish, the loss of BMP2 activity leads to a much more severe phenotype: deficiencies in ventral structures are observed in *swirl* mutant embryos with a concomitant expansion of dorsal structures (Mullins et al., 1996). Moreover, the expression pattern of *bmp2* in mouse embryos is also different from that of zebrafish. In mice, *bmp2* is expressed in the extraembryonic mesoderm and precardium (Lyons et al., 1989, 1990 and 1995), whereas, in zebrafish, *zbmp2* expression in the heart anlage and the heart proper is not detectable (Nikaido et al., 1997; our data, not shown). We conclude that *swirl* mutants lack blood and heart precursors (Fig. 5) because these fates do not exist in mutant embryos.

The *swirl* mutant phenotype more closely resembles the phenotype of BMP4 knock-out mice (Winnier et al., 1995), and the similarity of expression patterns of zebrafish *bmp2* and *Xenopus bmp4* has been interpreted to mean that zBMP2 is functionally the equivalent of *Xenopus* BMP4 (Nikaido et al., 1997). BMP4 homozygous mutant mouse embryos die early, show disorganized mesoderm and strongly truncated posterior structures (Winnier et al., 1995). This supports the idea that zBMP2 resembles mBMP4, rather than mBMP2, in function.

We have considered the possibility that, given

the high similarity between zBMP2 and zBMP4, these genes were named incorrectly. Within the C-terminal mature domains, the identity between zebrafish and mouse BMP2 is only 2% higher than that between zBMP2 and mouse BMP4 (zBMP2-mBMP2, 83%; zBMP2-mBMP4, 81%). However, the differences between amino acid sequence identities in the pro-domains are higher (zBMP2-mBMP2, 54%; zBMP2-mBMP4, 47%) (Nikaido et al., 1997). In humans, mouse, *Xenopus* and zebrafish, amino acid residues can be found that are specific for BMP2 and BMP4, respectively. For example, the two amino acids next to the RXKR processing site are Gln Ala in BMP2, but Ser Pro in BMP4. By comparing the protein sequences of zebrafish and mouse BMP2/4, we found that in 25 cases there were amino acids present in both BMP2 proteins, while at the same time there was a different pair of amino acids present in both BMP4 proteins. There was not a single case where an amino acid present in zBMP2 was present in mBMP4, while a different pair of amino acids was to be found in zBMP4 and mBMP2. Therefore, we believe that *zbmp2* and tetrapods *bmp2* stem from a common ancestor. Originally, both *bmp2* and *bmp4* ancestors might have acted as signaling molecule to specify ventral structures. In the course of evolution, however, different BMPs have taken up different functions, exemplified by the fact that mouse BMP4 and zebrafish BMP2 pattern the mesoderm in the early gastrula, while mouse BMP2 does not function there. It is also possible that more BMP2-like paralogues will be found in mouse or fish.

Conservation of the BMP/Chordin system

We have recently shown that lack of *chordino* affects the transcriptional regulation of both *chordino* and *zbmp4* (Hammerschmidt et al., 1996a; Schulte-Merker et al., 1997), and that interfering with the BMP-signaling pathway has severe effects on *chordino* transcriptional regulation (Schulte-Merker et al., 1997). Here we show that transcript levels of both *zbmp2* and *zbmp4* are downregulated in *swirl* mutant embryos (Fig. 6). This provides more evidence for our previous model, where Chordin and BMPs not only interact on the protein level, but also set up a balance of Chordin activity (high dorsally) and BMP activity (high ventrally), which, if disturbed, regulates itself: The loss of *swirl* activity leads to downregulation of zBMP2 and zBMP4 levels, while a loss of *chordino* function causes expansion of the expression domains of *zbmp2* and *zbmp4*, respectively.

Our findings presented here provide genetic evidence that the Chordin-BMP interaction establishes the dorsal-ventral
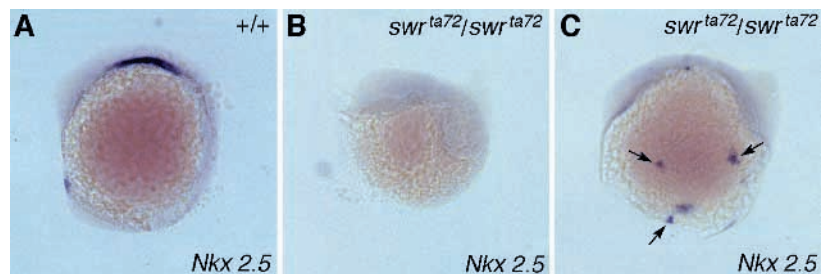

Fig. 5. *swirl* mutant embryos fail to form heart structures, as detected by in situ hybridization with *Nkx 2.5* markers (A-C; 15 somite stage, lateral view). Arrows in C point to a few ectopically placed cells positive for *Nkx 2.5*.
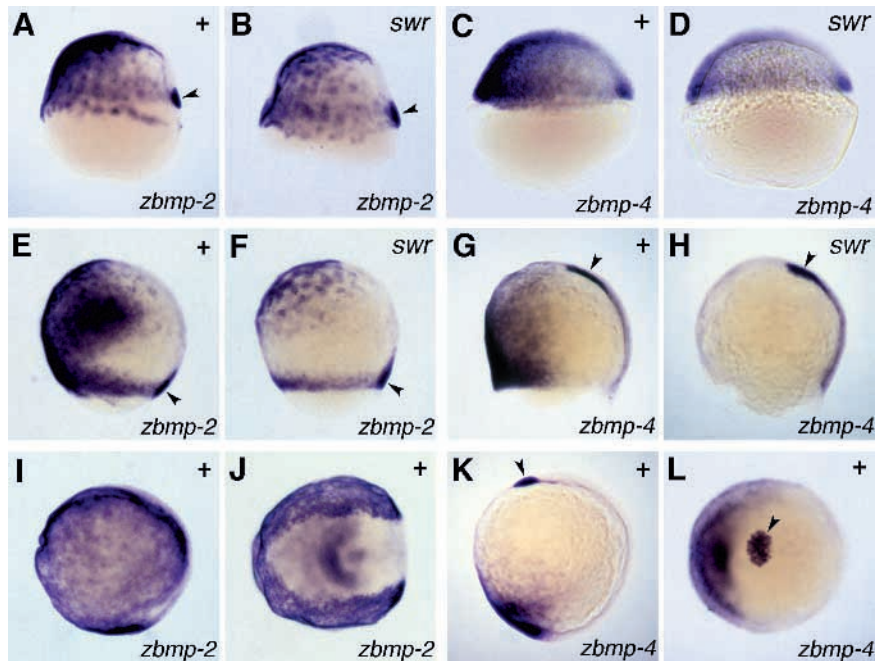
Fig. 6. Maintenance, but not induction, of both *zbmp2* and *zbmp4*, is affected in *swirl* mutant embryos. In situ hybridization with *zbmp2* (A-B,E-F,I-J) and *zbmp4* (C-D,G-H,K-L) antisense probes. Stages analyzed were 60% epiboly (A-B), 50% epiboly (C-D), 80% epiboly (E-H), and tailbud stage (I-L). Animal pole is up and dorsal is to the right, except in J and L, which are animal pole views. Arrowheads in A,B,E-H,K and L point at dorsal expression of *zbmp2* and *zbmp4*.

axis of zebrafish embryos. We propose that common mechanisms are used for dorsal-ventral pattern formation in *Drosophila*, *Xenopus* and zebrafish.

Are BMP2 and BMP4 redundant?

In ectopic expression studies in amphibian and fish embryos, BMP2 and BMP4 both promote ventral specification of the mesoderm and prevent neural specification in the ectoderm. This has led to speculation that the functions of BMP2 and BMP4 in the regulation of early dorsoventral pattern formation might be redundant. Here we show that in fish this is not the case, and that deficiencies in the zebrafish *bmp2* gene lead to a severe dorsalization of the embryo in both the mesoderm of the marginal zone and the ectoderm of the animal zone indicating that BMP4 cannot compensate for the loss of BMP2 in dorsoventral patterning. This is consistent with the expression patterns of *zbmp2* and *zbmp4* in wild-type and *swirl* mutant embryos. In animal ventral regions of wild-type embryos, the expression of *zbmp2* is maintained during the course of gastrulation, while *zbmp4* is not expressed, indicating that at this stage, zBMP2 is the only ventralizing agent. In marginal ventral regions, *zbmp2* and *zbmp4* are coexpressed, however, *zbmp4* expression depends on zBMP2, as indicated by the reduced initial expression and subsequent loss of the marginal *zbmp4* expression in *swirl* mutant embryos. Thus, *swirl* mutant embryos appear to contain only little if any ventralizing activity in the form of zBMP4 from its initial, possibly zBMP2-independent expression at pregastrula stages. This notion is consistent with the previously reported expression of the ventral marker gene *eve1*, which is present at reduced levels in *swirl* mutant embryos of early gastrula stages, but not detectable during later stages of gastrulation (Mullins et al., 1996).

swirl function is not essential after gastrulation

We have been able to completely rescue the *swirl* mutant phenotype. Homozygous mutant adults were obtained that appeared normal in all aspects analyzed thus far. Interestingly,

while BMP2 has been implied to have a function in a variety of processes such as fin development, bone formation, brain development and others (reviewed by Hogan, 1996), in fish, the activity of zBMP2/*swirl* is not essential for any of these processes. Also, while it has been often speculated that either BMP2 or BMP4 plays a role as the initial inducer of ventral mesoderm, we have failed to find evidence supporting this notion: embryos derived from a homozygous mutant *swirl* mother do not show defects in ventral mesoderm specification. Therefore, it appears that BMP2 activity is only required during gastrulation, but can be compensated for or is not essential during later stages of life.

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