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BMP signalling regulates anteroposterior endoderm patterning in zebrafish

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Abstract

In vertebrates, the embryonic dorsoventral asymmetry is regulated by the bone morphogenetic proteins (Bmp) activity gradient. In the present study, we have used dorsalized *swirl* (*bmp2b*) and ventralized *chordino* (*chordin*) zebrafish mutants to investigate the effects of dorsoventral signalling on endoderm patterning and on the differentiation and positioning of its derivatives. Alterations of dorsoventral Bmp signalling do not perturb the induction of endodermal precursors, as shown by normal amounts of cells expressing *cas* and *sox17* in *swirl* and *chordino* gastrulae, but affect dramatically the expression pattern of *her5*, a regulator of endoderm anteroposterior patterning in zebrafish. In particular, increased levels of Bmp signalling in *chordino* gastrulae are associated with a markedly reduced *her5* expression domain, that may be abolished by injecting *bmp2b* mRNA. Conversely, in *swirl* mutants, lacking Bmp2b, the *her5* expression domain is expanded. Thus, a gradient of Bmp2b signalling defines the extension of the *her5* expression domain at gastrulation and the allocation of anterior endodermal precursors. A balanced Bmp2b signalling is also required for the normal development of the pancreas, as shown by the sharp reduction of the pancreatic primordium in *swirl* embryos and its expansion in *chordino* mutants. In the latter, at 3 days post-fertilization, the increased Bmp signalling does not compromise the endocrine/exocrine pancreas compartmentalization, but the right/left positioning of the pancreas and liver is randomized. Our results suggest that by regulating the expression of *her5*, the Bmp2b/Chordin gradient directs the anteroposterior patterning of endoderm in zebrafish embryos. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Zebrafish; Embryo; Development; Endoderm; Dorsoventral patterning; Anteroposterior patterning; Bmp2b; Chordin; *swirl*; *chordino*; *Casanova*; *sox17*; *her5*; Gut; Pharynx; Endocrine pancreas; Exocrine pancreas; Liver; Chirality; In situ hybridization; Microinjection; Morpholino

1. Introduction

The mechanisms underlying early endoderm patterning in vertebrates are still much less understood than those controlling ectoderm and mesoderm induction and regionalization. It is well known that signals arising from the notochord and other adjacent structures are essential for the development of endodermal organs, but the mechanisms involved in early pre-patterning of the endoderm during gastrulation are only beginning to be disclosed (Bally-Cuif et al., 2000; Cleaver and Krieg, 2001).

In zebrafish, endoderm induction relies on Nodal signalling (Peyrieras et al., 1998; Alexander and Stainier, 1999; David and Rosa, 2001) leading to the expression of markers, such as *cas* and *sox17* (Aoki et al., 2002; Dickmeis et al., 2001; Kikuchi et al., 2001), which are, however, expressed in all endodermal precursors and, thus, do not confer a regional pre-patterning to these cells. So far, the only identified regionalized endodermal marker is *her5*, a hairy/enhancer of split-related transcriptional regulator (Fischer and Caudy, 1998), which is expressed at gastrulation in a subset of endodermal precursors and controls the cell contribution along the anteroposterior axis of endoderm (Bally-Cuif et al., 2000).

The fate mapping studies of Warga and Nusslein-Volhard (1999) suggest that during early gastrulation in zebrafish, the endodermal precursors are pre-patterned in such a way that those situated more dorsally originate anterior structures, while the ventrolateral ones give rise to posterior derivatives. On this basis, we hypothesized that dorsoventral signals may play a role at gastrulation in the early patterning of the endoderm. Such assumption was also supported by the idea that dorsalizing factors are implicated in early endoderm patterning in amphibians (Henry et al., 1996; Sasai et al., 1996).

In zebrafish, as in *Xenopus*, the dorsoventral asymmetry of the embryo is determined by a gradient of Bmp activity resulting from the action of the ventralizing Bmps antagonized by dorsalizing factors such as Chordin (Piccolo et al., 1996). This prompted us to address whether *bmp2b* and *chordin* are involved in endoderm patterning in zebrafish embryos. For this purpose, we have analyzed the distribu-

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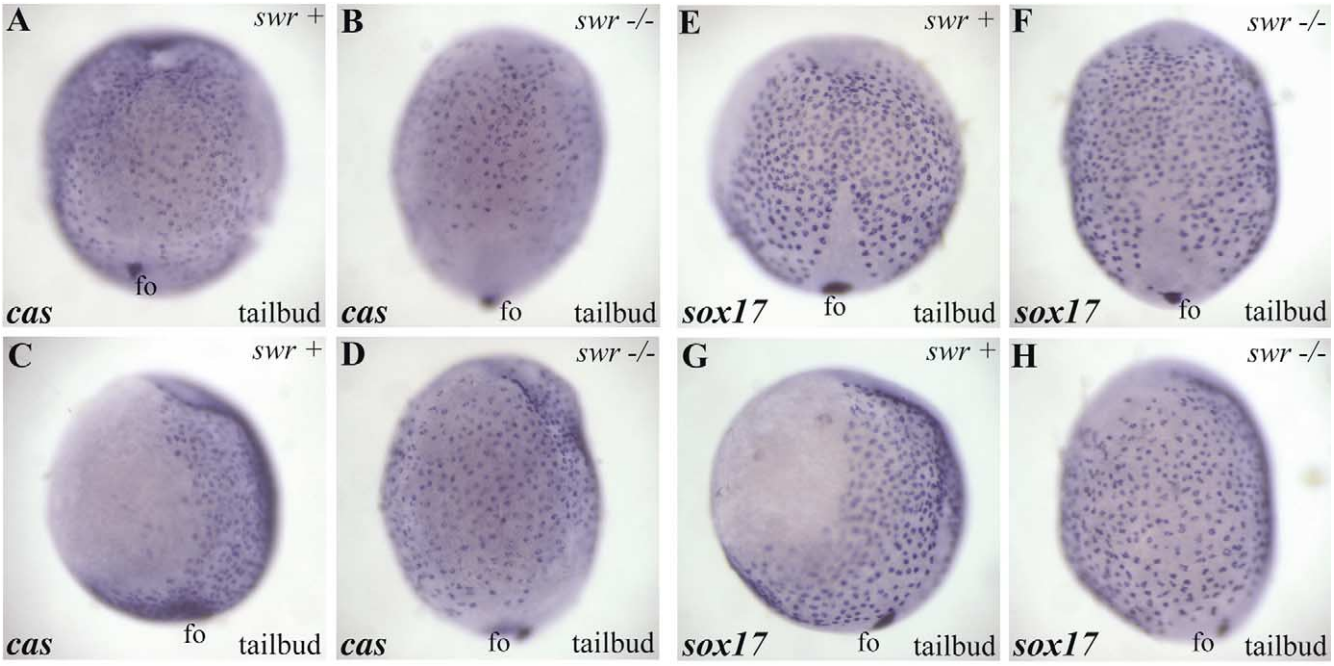

Fig. 1. *cas* and *sox17* expression in *swirl* embryos at the tailbud stage. *cas* and *sox17* are expressed in endodermal precursors (blue spots) and in non-involuting forerunner cells (fo). A, B, E and F are dorsal views with anterior to the top. C, D, G, and H are lateral views with dorsal to the right. Lateral views show a delay in convergence of endodermal precursors in the mutants. The *swirl* mutants are easily recognized owing to their elongated shape.

tion of endodermal precursors at gastrulation and the patterns of genes expressed in endodermal derivatives of dorsalized (*swirl/bmp2b*) and ventralized (*chordino/chordin*) zebrafish mutants (Mullins et al., 1996; Hammerschmidt et al., 1996; Kishimoto et al., 1997; Miller-Bertoglio et al., 1997; Schulte-Merker et al., 1997).

We found that the dorsoventral Bmp gradient does not influence the induction of endodermal precursors but affects markedly the extension of the *her5* expression domain. In particular, the level of *bmp2b* activity is inversely related to the number of *her5*-expressing cells and affects severely the size and position of endodermal derivatives. Our results strongly suggest that in zebrafish the Bmp gradient controls the endoderm anteroposterior patterning by regulating the expression of *her5*.

2. Results and discussion

2.1. Analysis of endoderm precursors at gastrulation in swirl and chordino mutants

During zebrafish endoderm formation *cas*, a sox-related gene, is the principal transcriptional effector of Nodal signalling and a potent inducer of the early endodermal marker *sox17* (Alexander and Stainier, 1999; Aoki et al., 2002; Dickmeis et al., 2001; Kikuchi et al., 2001). Hence, as a first step to examine the role of Bmp2b/Chordin gradient on endoderm formation we have investigated by in situ hybridization the embryonic expression patterns of the *cas* and *sox17* genes in *swirl* and *chordino* mutants. It has been shown that in the gastrula *cas* expression precedes but behaves essentially as *sox17* expression (Kikuchi et al., 2001). Accordingly, at the tailbud stage in embryos exhibiting the normal phenotype, the expression patterns of *cas* and *sox17* were superimposable, being detected in endodermal precursors and in non-involuting forerunner cells (Fig. 1, compare A and C with E and G). The number and distribution of *cas*- and *sox17*-positive cells were indistinguishable in all siblings from heterozygous *chordino* parents (data not shown). Moreover, a comparable number of *cas*- and *sox17*-positive cells was also present in both mutants (229 ± 9 cells on left side, $n = 3$) and normal siblings (211 ± 15 cells on left side, $n = 3$) from heterozygous *swirl* fish, although the convergence of endodermal precursors was slightly delayed in the mutants (Fig. 1, compare C with D and G with H). Hence the Nodal signalling pathway, which leads to the induction of endodermal precursors, is substantially unaffected by an unbalanced gradient of dorsoventral signals, although the dorsalized mutants display a defect of cell convergence. In this regard, the convergent and extension movements during zebrafish gastrulation were recently shown to be regulated by the gradient of Bmp activity (Myers et al., 2002). In particular, it was found that low levels of Bmp activity promote extension with little convergence in all regions, a finding well correlated with the delayed convergence of endodermal precursors in *swirl* mutants observed in the present study.

Although the above results suggested that the dorsoventral Bmp gradient does not affect quantitatively the forma-

tion of endodermal precursors, repercussions on the expression of regionalized endodermal markers could not be ruled out. In this respect, we considered the embryonic expression of *her5*, which is normally expressed during gastrulation in a subset of presumptive endodermal/mesendodermal cells and in the presumptive mid-hindbrain (Bally-Cuif et al., 2000, and Fig. 2A,C,E). As shown in Fig. 2B,D,F at the same stage, the endodermal *her5* expression domain was markedly reduced in *chordino* mutants and sharply increased in *swirl* embryos, in which the *her5*-positive cells were distributed in a broad band all around the gastrula. These observations suggested that the number of *her5*-positive cells was inversely correlated to the level of Bmp2b activity. To test this hypothesis, the *bmp2b* mRNA was overexpressed in wild type embryos in order to obtain a hyperventralized phenotype. Indeed, as shown in Fig. 3, the *her5* expression domain was further reduced or even completely abolished in such hyperventralized embryos.

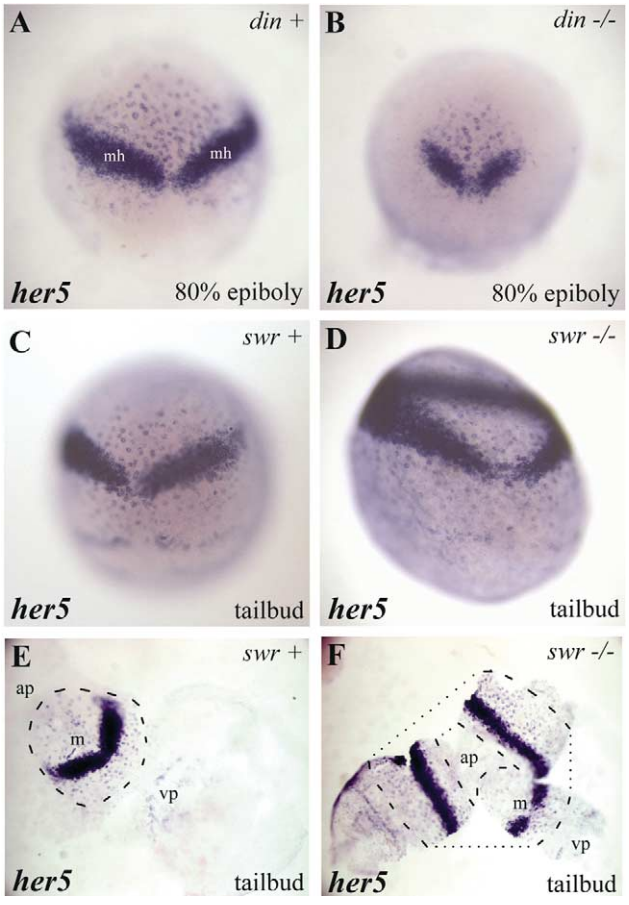

Fig. 2. *her5* expression is markedly altered in *chordino* and *swirl* mutants. In normal embryos (A, C and E), *her5* is expressed in a subset of presumptive endodermal/mesendodermal cells (blue spots) and in the presumptive mid–hindbrain (mh, dark regions). The *her5* expression domain is reduced in *chordino* mutants (B) and expanded in *swirl* mutants (D,F). (A–D): dorsal views with anterior to the top; (E,F): flat-mount views in which the dashed lines delimit the *her5* endodermal domain shown to encircle the entire gastrula in *swirl* mutants (F). ap, animal (anterior) pole; vp, vegetal (posterior) pole; m, midline.

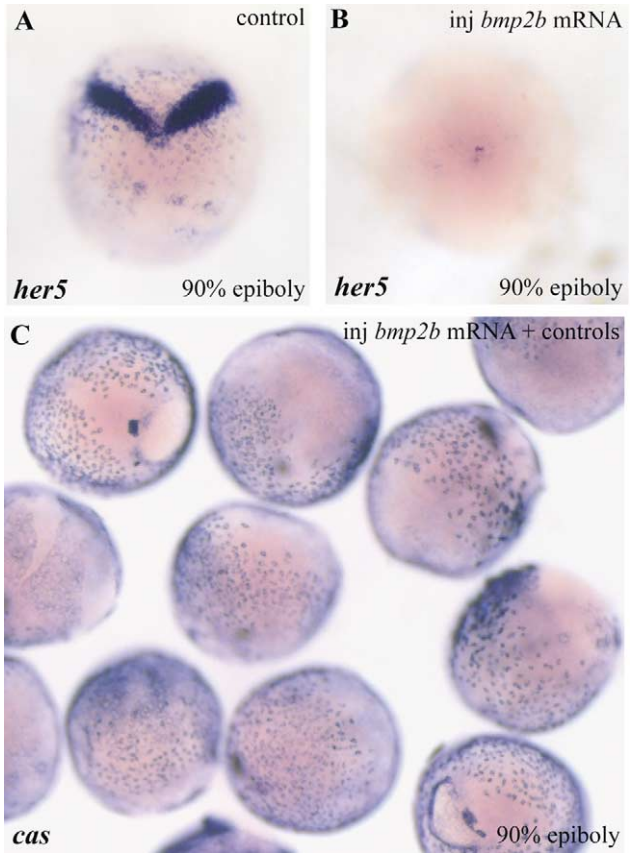

Fig. 3. *cas* and *her5* expression in zebrafish embryos injected with *bmp2b* mRNA. The *her5* expression domain is dramatically reduced in hyperventralized embryos injected with *bmp2b* mRNA (B), compared to controls injected with an unrelated mRNA (A). (C) When hyperventralized and control gastrulae are mixed and hybridized with the *cas* probe, they are indistinguishable. A and B are dorsal views, with anterior to the top.

The *bmp2b* overexpression inhibited *her5* expression, but did not affect significantly the induction of endodermal precursors, as evidenced by the distribution pattern of *cas*- and *sox17*-positive cells (shown for *cas* in Fig. 3C).

We next investigated whether other Bmp proteins, in addition to Bmp2b, could also contribute to the regulation of the genes expressed in endodermal precursors. For this purpose, embryos obtained from heterozygous *swirl* parents were injected with an antisense morpholino against the *alk8* messenger, encoding a type I transforming growth factor beta (TGFβ) receptor whose inactivation blocks the transduction of Bmp signals (Payne et al., 2001; Bauer et al., 2001; Mintzer et al., 2001). As a result of the morpholino injection, all embryos displayed the *swirl* phenotype in terms of *cas*, *sox17* and *her5* expression patterns (data not shown). The finding that the phenotype obtained by *alk8* inactivation was indistinguishable from that of *swirl* mutants provides an indirect evidence that endogenous levels of other Bmps do not compensate significantly the regulatory activity of *bmp2b* on *her5* expression in these mutants.

Altogether, the above results indicate that dorsoventral

signals (Bmp/Chordin) do not affect endoderm specification, as evidenced by the maintenance of normal amounts of endodermal precursors in ventralized, hyperventralized and dorsalized embryos. Conversely, the perturbation of dorsoventral signals affected dramatically the *her5* expression pattern. In particular, we found that the extension of the endodermal *her5* expression domain decreased progressively as the Bmp2b activity increased in dorsalized, normal, ventralized and hyperventralized embryos, respectively. We therefore conclude that in zebrafish the Bmp2b/Chordin activity gradient plays an essential role in the regulation of *her5* expression.

2.2. Analysis of the developing gut in swirl and chordino mutants

In order to assess the impact of the altered *her5* expression in the mutants, we have analyzed gut differentiation in further developmental stages of *swirl* and *chordino* embryos. For this purpose, we checked the embryonic expression of *fkd7/foxa1*, a member of the class I *fork head* domain gene family, expressed in the hypochord, the ventral neural tube and the developing gut (Odenthal and Nusslein-Volhard, 1998). *swirl* mutants were analyzed only up to the 12- to 14-somite stages, since after that period they start to die. We found that in *swirl* mutants, in comparison to phenotypically normal embryos, the *fkd7*expression domain was enlarged in the anterior gut, whereas *fkd*-positive cells were strikingly reduced in number or even absent in the posterior endoderm (Fig. 4, compare A and C with B and D). On the contrary, in *chordino* mutants, the anterior gut region was slightly reduced whereas the posterior gut was clearly expanded (Fig. 4, compare E and G with F and H).

To better evaluate the formation of the anterior gut region of *chordino* embryos, we checked the embryonic expression of *col2A1* (Yan et al., 1995), a type II collagen gene whose expression provides an indication of the developmental status of the pharyngeal arches in zebrafish embryos (Piotrowski and Nusslein-Volhard, 2000). The expression pattern of this marker, analyzed at 1 day post-fertilization, indicated a marked reduction of the pharyngeal cartilage in *chordino* mutants compared to normal siblings (Fig. 4, compare I with J). This reduction of the pharyngeal cartilage is likely to reflect a decrease in the amount of pharyngeal endoderm, a condition which is reminiscent of the phenotypes of *casanova*, *one-eyed pinhead* and *van gogh* mutants, all characterized by endoderm defects perturbing the formation of the pharyngeal cartilage (Piotrowski and Nusslein-Volhard, 2000).

Taken together, our data suggest that the alteration of the *her5* expression domain by dorsoventral signals modifies the recruitment of endodermal cells to the anterior and posterior regions of the developing gut. In this respect, it has been proposed that *her5*-positive cells participate to the formation of anterior endodermal derivatives such as the pharynx (Bally-Cuif et al., 2000). Such hypothesis is consis-

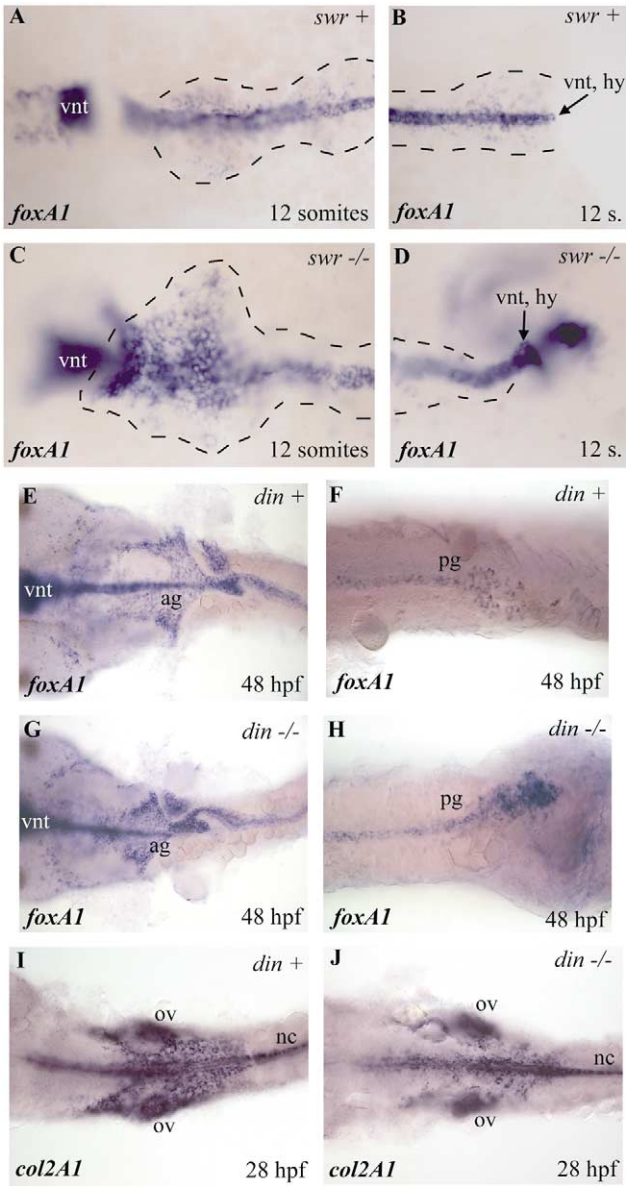

Fig. 4. The development of the anterior and posterior regions of the gut is markedly affected in *swirl* and *chordino* mutants. Anterior (A) and posterior (B) regions of a control embryo at the 12-somite stage evidencing the *fkd7/foxa1* expression in endodermal cells spread on both sides (delimited by a dashed line) as well as along the midline in the overlapped hypochord (hy) and ventral neural tube (vnt). In a *swirl* mutant at the same stage, the endodermal expression domain of *fkd7/foxa1* is markedly enlarged anteriorly (C). Conversely, in the twisted caudal region the number of endodermal *fkd7/foxa1*-positive cells is much reduced, while the labelling in the hypochord and ventral neural tube is strongly enhanced (D). At 48 hpf, the extension of the anterior gut (ag) is reduced in a *chordino* mutant (G) compared to a normal embryo (F), whereas in the posterior gut (pg) the number of *fkd7/foxa1*-positive cells is much higher in the *chordino* mutant (H) than in the normal embryo (G). In a *chordino* mutant at 28 hpf (J), *col2A1* expression is reduced in the pharyngeal cartilage but similar in the otic vesicle (ov) compared to the control (I). All pictures are ventral views, with anterior to the left. nc, notochord.

tent with the observation that in *swirl* mutants, in which the number of *her5*-positive cells was increased, the anterior gut was enlarged. On the contrary, in *chordino* mutants, which possess few *her5*-positive cells, the anterior gut was reduced, particularly in the pharyngeal region.

2.3. Analysis of pancreas precursors in swirl and chordino mutants

The above results indicated that dorso-ventral signals influence significantly the development of the anterior and posterior regions of the gut. To address whether the development of derivatives of the central division of the gut were also affected we focused our attention on the pancreas, an organ asymmetrically located on the right side of the body by late embryogenesis.

In order to identify the pancreatic primordium, we checked the expression of *neuroD* and *islet-1*, two transcription factors expressed in the early pancreas (Korzh et al., 1998; Biemar et al., 2001). Both markers evidenced a dramatic reduction of pancreatic precursors in *swirl* mutants (shown for *neuroD* in Fig. 5B), whereas in *chordino* mutants the pancreatic primordium was enlarged (shown for *islet-1* in Fig. 5D,F). Dramatic changes in *swirl* mutants and light differences in *chordino* embryos are in agreement with their strong and mild phenotypes, respectively (Mullins et al., 1996; Hammerschmidt et al., 1996). The striking reduction of both the posterior endoderm and pancreatic precursors in *swirl* embryos suggests that in zebrafish the pancreatic primordium possesses a posterior identity. Nonetheless, though drastically reduced in number, the pancreatic precursors are not missing at this stage in *swirl* embryos. This may be due to other Bmp-like activities compensating the lack of Bmp2b in such mutants at early or late stages of development. Otherwise, it can also be hypothesized that the pancreas is a borderline structure between the intermediate and posterior regions of the endoderm, so that some pancreatic precursors are in any case present in dorsalized *swirl* mutants, in spite of the reduced posterior gut. Moreover, endodermal organs asymmetrically located in the adult, such as the pancreas and liver, originate from both dorsal and ventral regions of the gastrula according to the endoderm fate map proposed by Warga and Nusslein-Volhard (1999). This dual origin may explain why a complete lack of pancreas is not observed in dorsalized *swirl* embryos.

The embryonic development of the pancreas was further analyzed in *chordino* mutants from 48 hpf, when the organ asymmetry is established, up to 3 days post-fertilization, using *insulin* and *trypsin* as markers of endocrine and exocrine cells, respectively (Argenton et al., 1999; Biemar et al., 2001). As shown in Fig. 6, despite some morphological alterations, the pancreas compartmentalization in *chordino* mutants appeared normal, with exocrine cells

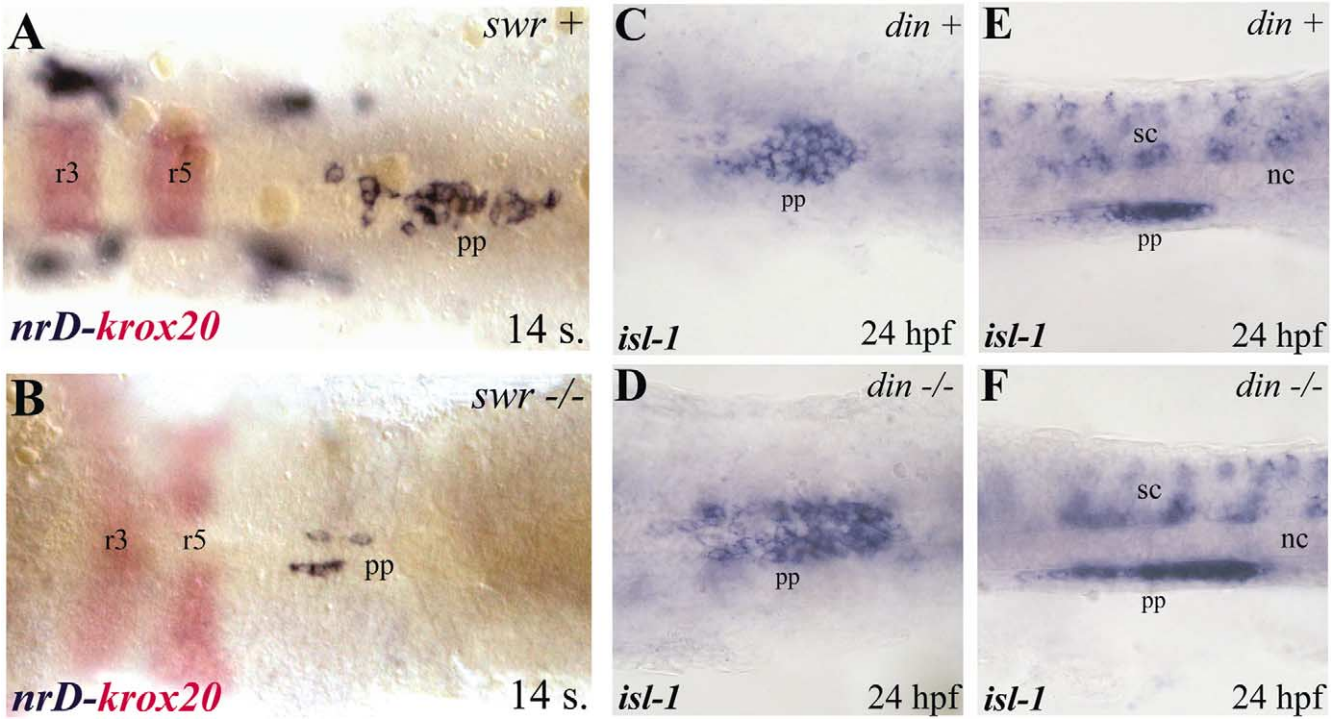

Fig. 5. Expression of early markers in the pancreatic primordium of *swirl* and *chordino* mutants. The expression of *neuroD* (*nrD*) in control (A) and *swirl* (B) embryos, at the 14-somite stage, shows that in the mutants the number of *neuroD*-expressing pancreatic precursors (pp) along the midline is drastically reduced and the lateral ectodermal staining is absent. *krox20* expression (red staining) in rhombomeres r3 and r5 has been used as a landmark. At 24 hpf, the expression of *islet-1* (*isl-1*) in control (C,E) and *chordino* embryos (D,F) illustrates a significant enlargement of the pancreas primordium in the mutants. *islet-1* is also expressed in a number of spinal cord (sc) cells. A–D: ventral views; E,F: lateral views; in all pictures anterior is to the left. nc, notochord.

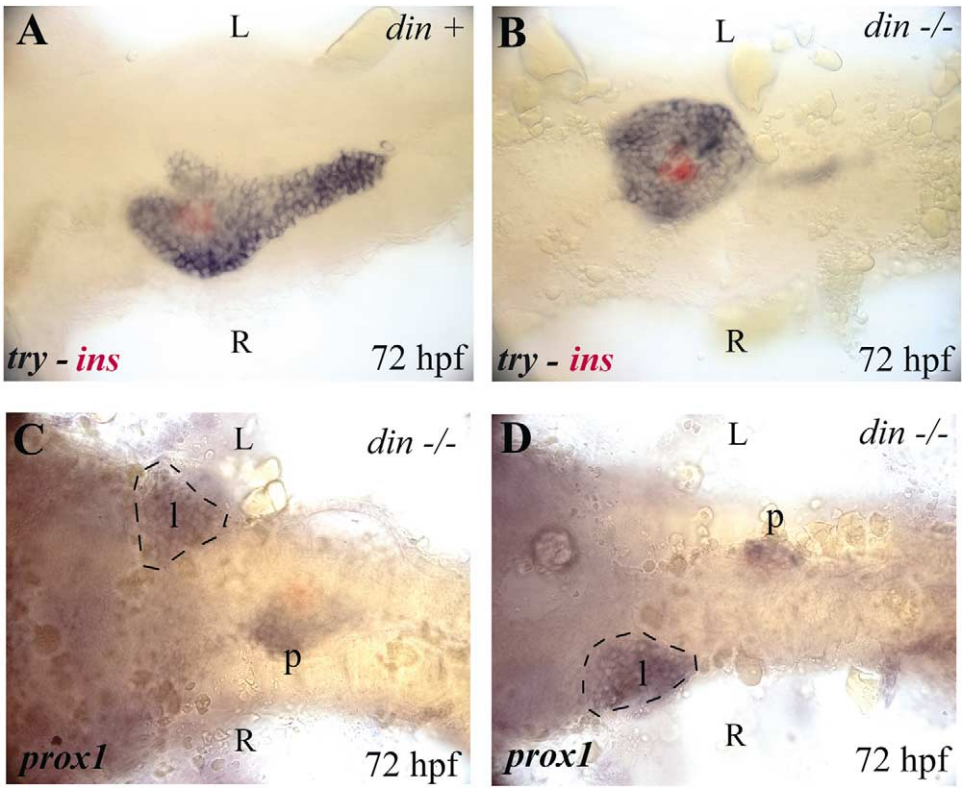

Fig. 6. Expression of pancreatic markers and randomization of embryonic pancreas and liver positioning in *chordino* embryos. A and B: *trypsin* (blue) and *insulin* (red) expression identify the exocrine and endocrine compartments of the pancreas, respectively, in a control (A) and a *chordino* (B) embryo. C and D: cells expressing *prox1*, identifying the liver (encircled by a dashed line), and *trypsin* are stained blue, those expressing *insulin* are stained red. The normal position of the pancreas on the right (A), may be either maintained (C) or reversed (B,D) in *chordino* mutants. The position of the liver is always opposite to that of the pancreas (C,D), so that when the position of the pancreas is reversed the liver is also misplaced (D). All pictures are ventral views with anterior to the left. L, left; R, right; l, liver; p, pancreas.

surrounding the endocrine islet, suggesting that early unbalanced Bmp signalling has no influence upon pancreatic cell fates. However, we found that *chordino* mutants exhibit randomization of the left/right positioning of both the pancreas and liver, the latter identified with the hepatic marker *prox1* (Glasgow and Tomarev, 1998) (Fig. 6, compare A with B, and C with D). Such randomization of organ position in an overactive Bmp signalling context, already observed by Chen et al. (1997) and Bisgrove et al. (2000), is well correlated with the finding that gut left–right asymmetry in *Xenopus* depends on the interaction between XLefty and BMP4 signalling (Branford et al., 2000). It thus appears that Bmp signalling is required in early embryonic stages for endoderm patterning and normal development of the pancreatic primordium. A balanced Bmp signal is also important to define a correct left–right asymmetry of the forming gut but does not interfere with the compartmentalization of the misplaced pancreas. The involvement of Bmp2b in the formation of the zebrafish pancreas is well correlated with the observations that Bmps promote the development of mouse pancreatic structures in vitro (Jiang et al., 2002), exert a mitogenic effect on pancreatic cancer cell lines and are highly expressed in human pancreatic tumors (Kleeff et al., 1999).

3. Conclusions

Our present results indicate that the early gradients of Bmp2b and Chordin at gastrulation contribute to the definition of the *her5* domain, thus regulating the anteroposterior endoderm patterning (Fig. 7). According to the endoderm fate map of Warga and Nusslein-Volhard (1999), the anteroposterior location of endodermal derivatives roughly corresponds to the dorsoventral position of their precursors in the late blastula. This study is the first attempt to provide a molecular explanation to this observation, showing that Her5 is a key factor implicated in the translation of the Bmp gradient into anteroposterior instructions that regionalize the zebrafish early endoderm.

In rodents, the bone morphogenetic protein BMP2 upregulates directly the expression of *Hes5*, by a Smad-mediated mechanism (Nakashima et al., 2001); similarly, it can be envisaged that in zebrafish, the *her5* expression may be regulated by Bmp2b through the Smad pathway. On the other hand, *her5* was shown to be repressed by a Notch-mediated intracellular signalling (Bally-Cuif et al., 2000) and, in addition, members of the TGF-β superfamily are known to regulate the expression of components of the Delta–Notch pathway in both vertebrates and invertebrates

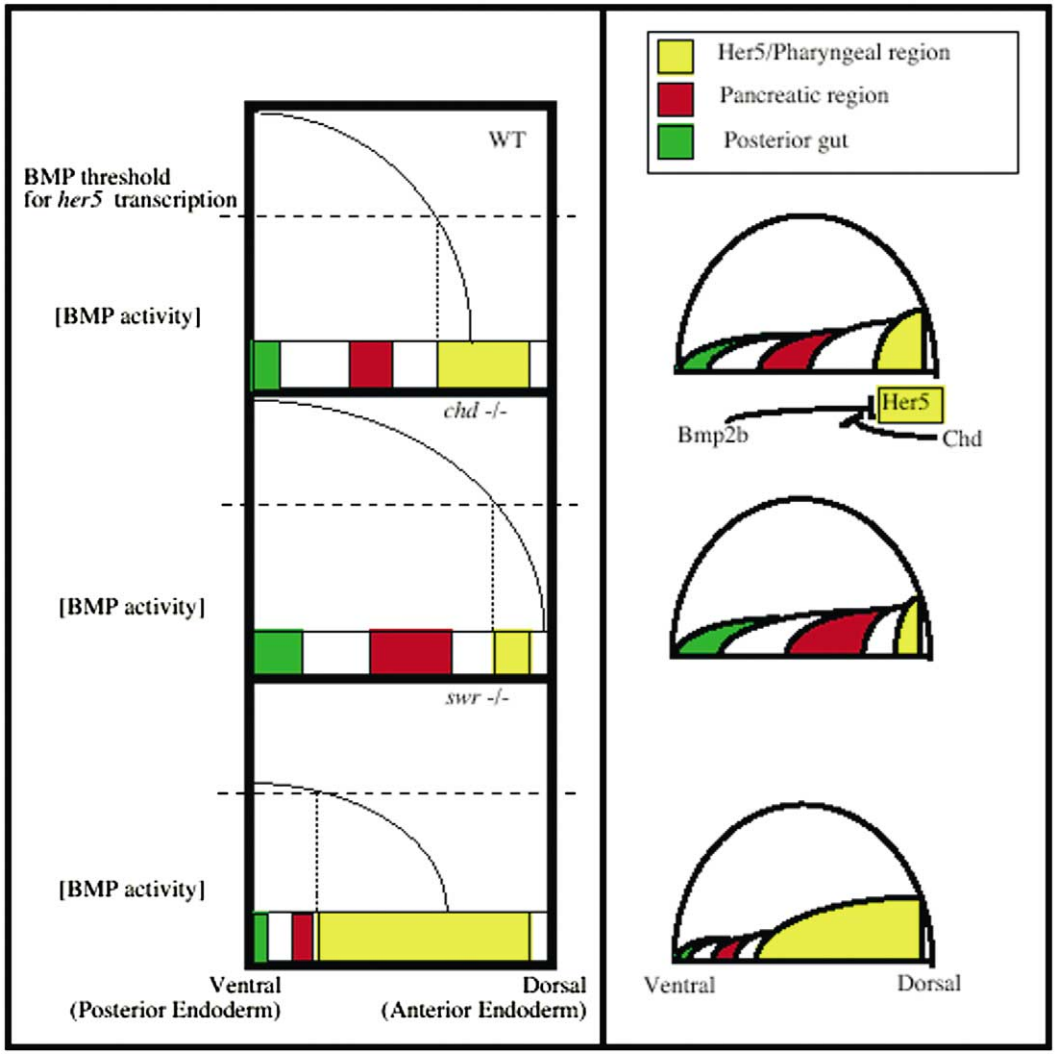

Fig. 7. Control of the extension of *her5* endodermal expression by the Bmp2b/Chordin antagonistic interaction. The inhibition of Chordin on Bmp2b activity relieves the negative effect of Bmp2b on the ventral boundary of *her5* expression. The different extensions of *her5* domain (Pharyngeal region), Pancreatic region and Posterior gut are depicted in the diagrams for wild type (top), *chordino* (middle) and *swirl* (bottom) embryos. On the left, relationships between Bmp activity gradient, posterior boundary of *her5* expression and relative proportions of endodermal domains are represented. On the right, interactions of *bmp2b*, *chordin* and *her5* affecting endoderm patterning at the blastula stage.

(Rauskolb and Irvine, 1999; Kim and Hebrok, 2001). Hence, the Bmp and Delta–Notch pathways may act either sequentially and/or in parallel in the regulation of *her5* expression. Notably, Bally-Cuif et al. (2000) postulated the occurrence of a local dorsal signal involved in the inhibition of Delta/Notch signalling and in the induction of *her5* expression; our results suggest that Chordin, as a dorsal secreted inhibitor of Bmp signalling, could be a candidate for such a role.

4. Experimental procedures

4.1. Zebrafish maintenance and mutant lines

Zebrafish (*Danio rerio*) were raised according to standard protocols (Westerfield, 1995). Homozygous *swirl* (swr^{ta72}) and *chordino* (din^{tt250}) mutants were obtained from natural matings between heterozygous parents. Embryos were staged by morphology during the first 24 h (Kimmel et al., 1995) and, subsequently, by time post-fertilization. Embryos to be analyzed at older stages (beyond 24 h post-fertilization) were raised in 2 mM 1-phenyl-2-thiourea medium to prevent pigmentation.

4.2. Microinjections

Injections were carried out during the first cell cycle. Messenger RNAs were synthesized using the mMessage mMachine in vitro transcription Kit (Ambion). *bmp2b* mRNA (100 pg/embryo) was co-injected with Green Fluorescent Protein (*GFP*) mRNA (50 pg/embryo) to assess the

distribution and quality of the injected messenger. Control embryos were injected with *GFP* mRNA alone.

In the experiment designed to block Bmp signaling, embryos were injected with 1 ng of either *alk8* morpholino or a negative control morpholino (GeneTools) in Danieau buffer.

4.3. Whole-mount in situ hybridization

Whole-mount in situ hybridizations were performed according to Thisse et al. (1993), using antisense riboprobes labelled with digoxigenin or fluorescein (Roche). Double staining was carried out according to Hauptmann and Gerster (1994). The following probes were used: *cas* (Dickmeis et al., 2001; Kikuchi et al., 2001; Aoki et al., 2001), *sox17* (Alexander and Stainier, 1999), *her5* (Bally-Cuif et al., 2000), *fkd7/foxa1* (Odenthal and Nusslein-Volhard, 1998) (MPMGp609C2234Q clone available at RZPD – Deutsches Ressourcenzentrum für Genomforschung GmbH), *col2A1* (Yan et al., 1995), *prox1* (Glasgow and Tomarev, 1998), *neuroD* (Korzh et al., 1998), *islet-1*, *insulin* and *trypsin* (Argenton et al., 1999; Biemar et al., 2001).

4.4. Microscopy and imaging

Following in situ hybridization, embryos were post-fixed in 4% paraformaldehyde and mounted in 85% glycerol/phosphate-buffered saline (PBS) for microscope observation. Embryos at gastrulation were cleared with a 2:1 mixture of benzylbenzoate:benzyl alcohol. At later stages, the yolk was dissected away. Observations were made with a Leica MZFLIII dissecting microscope equipped for epifluorescence and a Leica DMR compound/Nomarski microscope and acquired with Leica DC200 and DC500 digital cameras. Image data were processed using the Adobe Photoshop 5.5 software.

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