Fgf signalling controls the dorsoventral patterning of the zebrafish embryo

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Summary

The establishment of dorsoventral (DV) patterning in vertebrate embryos depends on the morphogenic activity of a group of Tgfβ superfamily members, the bone morphogenetic proteins (Bmps) (which specify ventral cell fates), and on their interaction with their dorsally secreted cognate inhibitors chordin and noggin. In the zebrafish, genetic analysis has revealed that Bmp2b and Bmp7, as well as their antagonist chordin, are required for proper DV patterning. The expression of Bmp genes is initially activated in the whole blastula. Well before the beginning of gastrulation, Bmp gene expression progressively disappears from the dorsal side to become restricted to the ventral part of the embryo. We show that this early restriction of Bmp gene expression, which occurs independently of noggin and chordin, is an essential step in the establishment of DV patterning. The progressive ventral restriction of Bmp gene transcripts is coincident with the spreading of Fgf activity from the dorsal side of the embryo, suggesting that Fgf signalling is implicated in dorsal downregulation of Bmp gene expression. In accordance with this, activation of the Fgf/Ras/Mapk-signalling pathway inhibits ventral Bmp gene expression, thereby causing a dorsalisation of the embryo. Conversely, inhibition of Fgf signalling causes Bmp gene expression to expand dorsally, leading to an expansion of ventral cell fates. In accordance with an important role of Fgf signalling in the DV patterning of the zebrafish, we show that loss of Fgf8 function enhances the ventralisation of chordin-deficient embryos. Our results thereby demonstrate that pre-gastrula stage Fgf-signalling is essential to delimit the expression domain of the genes encoding the functional morphogen of the dorsoventral axis of the early zebrafish embryo.

Key words: Zebrafish, Dorsoventral patterning, Fgf, Bmp, Sprouty 2

Introduction

In the zebrafish, genetic studies have demonstrated that the activity of Bmp2b and Bmp7 is required to establish the morphogenic activity gradient that specifies cellular identity along the dorsoventral (DV) axis of the early embryo (Kishimoto et al., 1997; Dick et al., 2000; Schmid et al., 2000). The secreted growth factor antagonists chordin and noggin directly bind Bmp proteins and abolish thereby their capacity to interact with their cognate Bmp receptors (Piccolo et al., 1996; Zimmermann et al., 1996). Loss-of-function of the Bmp antagonist chordin causes excess Bmp activity, leading thereby to an expansion of ventral cell fates (Schulte-Merker et al., 1997; Miller-Bertoglio et al., 1999a). This observation demonstrates that a modulation of Bmp protein activity is required for proper DV patterning.

As soon as the zygotic genome becomes activated, the expression of the genes encoding the DV morphogen, *bmp2b* and *bmp7*, is initiated throughout the embryo. However, as development proceeds, the expression of *bmp2b* and *bmp7* becomes very rapidly restricted to the ventral part of the embryo where Bmp proteins actually specify ventral cell fates. This restriction of Bmp gene expression occurs well before the beginning of gastrulation and is therefore one of the first major manifestations of zygotic DV patterning. Surprisingly, relatively little attention has been devoted to the functional relevance of the mechanisms that act to ensure the early confinement of Bmp gene expression to the ventral side of the embryo. The homeobox transcription factor bozozok (Boz) is expressed in the dorsal-most cells of the marginal blastoderm where it directly represses *bmp2b* transcription (Koos and Ho, 1997; Leung et al., 2003). Considering its very small domain of expression, Boz can however clearly not by itself be responsible for the progressive downregulation of *bmp2b* expression throughout the entire dorsal half of the embryo.

We show that the early restriction of Bmp gene expression is independent of the well-known Bmp antagonists chordin (Chd) and noggin (Nog) that bind Bmp proteins and prevent them from interacting with Bmp receptors. We show that the early restriction of Bmp gene expression depends on fibroblast growth factor (Fgf) activity that progressively spreads from the dorsal side of the embryo, causing Bmp gene expression to recede. In accordance with a role of Fgf/Ras/Mapk signalling in the DV patterning of the early zebrafish embryo, activation of this pathway abolishes expression of the ventralising Bmp genes, causing thereby a dorsalisation of the embryo. Most importantly, we show that inhibition of Fgf signalling causes

Bmp gene expression to expand dorsalwards, leading to a severe expansion of ventral cell fates. Through the analysis of the genetic interactions between *fgf8* and *chd*, we further demonstrate that these two factors cooperate in vivo to ensure the proper DV patterning of the embryo. Our study therefore shows that in addition to inhibition of Bmp protein activity by Bmp-binding antagonists, Fgf-mediated restriction of Bmp genes expression is essential for the establishment of the zebrafish DV axis.

Materials and methods

Cloning of zebrafish sprouty 2

Zebrafish sprouty 2 (*spry2*) was amplified by RT-PCR based on the sequences of the ESTs fk93e11 (GenBank BE556991) and fk66h11 (GenBank BE016191). The cDNA sequences for zebrafish *spry2* has been submitted to GenBank (AY285923).

RNA microinjection

The zebrafish Spry2 open reading frame was inserted into the *Bam*HI/*Xho*I sites of pCS2+. Through PCR-based mutagenesis, a Spry2 construct was generated in which the target sequence of the anti-spry2 morpholino (ATGGAGACGAGAACTCAAAATGGCG) was converted into ATGGAGACCCGTACTGAAAATGGCG, rendering the cDNA insensible to morpholino-based translational inhibition. The open reading frames of Fgf24 and Erm1 were cloned into the *Eco*RI/*Xba*I sites and Pea3 into the *Bam*HI/*Xba*I sites of pCS2+. For the DN-Fgfr1 construct, the extracellular and transmembrane parts of the zebrafish Fgfr1 were cloned in front of a stop codon into the *Eco*RI/*Xho*I sites of pCS2+ vector. For sense RNA synthesis, Fgf24-, Pea3-, Erm1- and DN-Fgfr1-pCS2+ were linearised using *Not*I; Spry2-pCS2+ was linearised using *Kpn*I. Constructs for microinjection of chordin (Miller-Bertoglio et al., 1999a), noggin 1 (Nog1) (Fürthauer et al., 1999), Fgf8 (Fürthauer et al., 1997), Fgf3 (Fürthauer et al., 2001), DN-Ras (Whitman and Melton, 1992) and mouse Spry2Y55F (Hanafusa et al., 2002) have been previously described. All injected RNAs have been synthesised with the mMessage Machine SP6 kit (Ambion). Injection was performed either into the yolk at the one-cell stage or in an animal blastomere at the 64-cell stage. Embryos were cultured at 28.5°C in Danieau 0.3× supplemented with 1% Penicillin/Streptomycin (Gibco, 15140-122). Except when specified otherwise, embryos were injected with the following doses of RNA: *fgf3*, 25 pg; *fgf8*, 0.2 pg; *fgf24*, 0.1 pg; *nog1*, 25 pg; *chd*, 200 pg; *erm1*, 100 pg; *pea3*, 100 pg; *spry2*, 25 pg; DN-Spry2, 200 pg; DN-Fgfr1, 500 pg; DN-Ras, 300 pg.

Morpholino injections

Antisense morpholino oligonucleotides (GeneTools, LLC) designed against *chd* (5′-ATCCACAGCAGCCCCTCCATCATCC-3′, 0.1 pmol), *spry2* (5′-CGCCATTTTGAGTTCTCGTCTCCAT-3′, 4 pmol) and *fgf8* (5′-GAGTCTCATGTTTATAGCCTCAGTA-3′, 0.4 pmol) were injected into two-cell stage embryos. Morpholinos were resuspended in 1× Danieau.

Whole-mount in situ hybridisation

For in situ hybridisation, the spry2 open reading frame was inserted into the *Bam*HI/*Xho*I sites of pBSKII+, the vector linearised with *Bam*HI and antisense RNA transcribed with T7 RNA polymerase. *bmp2b*, *cyp26a*, *fgf8*, *foxi*, *ved* and *zic2* were isolated in the course of a large-scale in situ hybridisation screen (http://zfin.org) and antisense RNA made through *Not*I linearisation and T7 transcription. The other clones used in this study have been previously described: *bmp7* (Schmid et al., 2000), *chd* (Miller-Bertoglio et al., 1999a), *draculin* (Herbomel et al., 1999), *en3* (Ekker et al., 1992), *fgf3* (Fürthauer et al., 2001), *fgf24* (Draper et al., 2003), *goosecoid* (Thisse et al., 1994), *hemoglobin* (Chan et al., 1997), *nog1* (Fürthauer et al., 1999), *otx2* (Mercier et al., 1995), *shh* (Krauss et al., 1993), *spry4* (Fürthauer et al., 2001) and *vhnf1* (Thisse and Thisse, 1999). All whole-mount in situ hybridisations were performed as described by Thisse and Thisse (http://zfin.org/zf_info/zfbook/chapt9/9.82.html).

For two-colour in situ hybridisation, embryos were incubated with a mixture of one digoxigenin- and one fluorescein-labelled probe. The digoxigenin-labelled probe was visualised using the standard in situ hybridisation protocol. The staining reaction was arrested by three 5 minute washes in 0.1 M glycine buffer (pH 2.2), 0.1% Tween20. After overnight incubation with a 1:5000 dilution of anti-fluorescein antibody (Roche, Ref. 1426338) the fluorescein-labelled probe was visualised using the ELF in situ hybridisation kit (Molecular Probes, Ref. E6604; used according to manufacturers instructions, except that the substrate component was diluted 80 times).

Pharmacological inhibition of Fgf signalling

To inhibit Fgfr activity, embryos were treated with SU5402 (Mohammadi et al., 1997) (Calbiochem) at 40 μM in 0.3× Danieau at 28.5°C in the dark.

Results

Regulation of pre-gastrula stage Bmp gene expression

In the zebrafish embryo, three genes encoding Bmps, *bmp2b*, *bmp7* and *bmp4*, are expressed at blastula and gastrula stages (Kishimoto et al., 1997; Nikaido et al., 1997; Dick et al., 2000; Schmid et al., 2000). *bmp2b* and *bmp7* are expressed first, at the sphere stage (4 hpf) soon after the beginning of expression of the zygotic genome, whereas *bmp4* transcripts become detectable about 1 hour later (30% epiboly). At sphere stage, expression of *bmp2b* and *bmp7* is detected throughout the blastoderm (Fig. 1A,D). Within the next 30 minutes, *bmp2b* transcripts disappear from the dorsalmost aspect of the blastula margin (not shown). This dorsal *bmp2b*-free zone then rapidly expands towards the ventral side and the animal pole (Fig. 1B) so that at the onset of gastrulation (shield stage, 6 hpf) *bmp2b* transcripts are excluded from the dorsal half of the non-marginal blastoderm (Fig. 1C). A similar dynamic disappearance from dorsal territories is observed for *bmp7* (Fig. 1D-F). At shield stage, *bmp2b* expression becomes moreover detectable at the dorsal margin (Fig. 1C). This marginal expression domain is unaffected in mutants disrupting DV patterning (Schmid et al., 2000), strongly suggesting that it is unrelated to the establishment of the DV polarity.

Because Bmps are known to control DV patterning, the restriction of their expression to the ventral domain of the blastula may be an essential step in the control of their morphogenic activity along the DV axis.

In *Xenopus*, the analysis of the *bmp4* promoter reveals that it contains Bmp-responsive elements, suggesting that *bmp4* expression is subject to positive autoregulation (Metz et al., 1998). Therefore, the progressive dorsal clearance of Bmp gene expression could be due to the action of dorsally secreted Bmp antagonists, such as Chd, which may progressively shut down Bmp gene expression through interference with the positive Bmp gene feedback loop. However, several observations argue strongly against such a model for the dorsal clearance of Bmp gene expression at blastula stages. First, the progressive ventral restriction of Bmp gene expression at blastula stages is unaffected in embryos in which Chd function

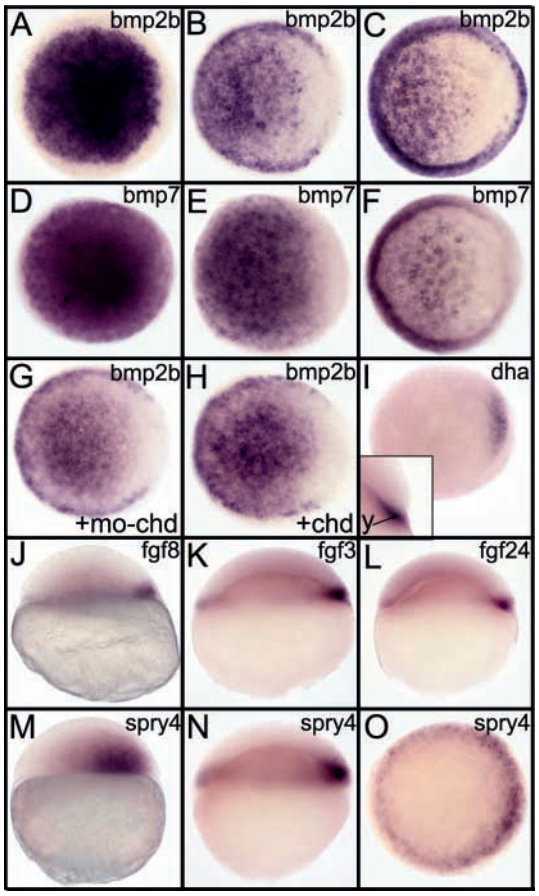

Fig. 1. Dorsal clearance of Bmp gene transcripts and concomitant formation of a dorsoventral Fgf gradient. (A-C) Expression of *bmp2b* at sphere stage (A), 30% epiboly (B) and at shield (C) stages. (D-F) Expression of *bmp7* at sphere (D), 30% epiboly (E) and at shield (F) stages. (G) Expression of *bmp2b* at 30% epiboly in embryo injected with a chd morpholino (mo-chd). (H) Expression of *bmp2b* at 30% epiboly in an embryo injected with chd RNA. (I) Expression of *dharma* (*dha*) in wild-type embryo at 30% epiboly (inset provides a lateral view of the same embryo). Expression of *dha* is restricted to the yolk syncytial layer (y). Expression of (J) *fgf8* at sphere stage, (K) *fgf3* at 30% epiboly and (L) *fgf24* at 30% epiboly. (M-O) Expression of *spry*4 at sphere (M), 30% epiboly (N) and late blastula (O) stages. Embryos are oriented dorsal towards the right. A-I,O are animal pole views; J-N are lateral views.

has been compromised through the injection of anti-Chd morpholinos (Fig. 1G) or the inactivating *chordino* mutation (not shown) (Schulte-Merker et al., 1997). Second, in the converse experiment, a Chd overexpression through mRNA microinjection, the expression pattern of *bmp2b* at early blastula stage is also unaffected (Fig. 1H). Only later, at gastrula stage, did embryos injected with *chd* RNA display a loss of Bmp gene expression from the ventral blastoderm (not shown). These findings show that Chd is not involved in the progressive restriction of Bmp gene expression to the ventral side of the zebrafish blastula. Similarly, overexpression of the Bmp antagonist Nog1 does not affect blastula stage *bmp2b* expression (Fig. 2F). Finally, blastula stage Bmp gene expression patterns are unaffected by the loss of Bmp2b or Bmp7 function in *swirl/bmp2b* and *snailhouse/bmp7* mutant embryos (not shown). Loss of Bmp gene transcripts in the ventral non-marginal blastoderm is observed only later, after the beginning of gastrulation (Schmid et al., 2000). All these observations argue strongly against a role for Bmp gene-autoregulation in the dorsal clearance of Bmp gene expression at the blastula stage.

The homeobox transcription factor dharma (Dha)/Boz acts as a repressor mediating the initial clearance of *bmp2b* expression from the dorsalmost aspect of the embryo (Koos and Ho, 1997; Shimizu et al., 2002; Leung et al., 2003). However, the dorsal Bmp gene-free zone becomes rapidly much larger than the *dha* expression domain which becomes restricted to the dorsal yolk syncytial layer by 30% epiboly (Fig. 1I). This shows clearly that the restriction of *bmp2b* expression can not be solely due to the cell-autonomous repressive activity of Dha and that another mechanism is involved in the control of early Bmp gene expression.

Analysis of a large set of gene expression patterns within the course of a whole-mount in situ hybridisation screen has allowed us to identify several Fgfs as candidate factors that could cause the progressive dorsal clearance of Bmp gene expression. Our analysis of the early expression pattern of *fgf3* (Kiefer et al., 1996), *fgf8* (Fürthauer et al., 1997) and *fgf24* (Draper et al., 2003) reveals that these genes are first expressed at the dorsal margin of the embryo at early blastula stage. Then, their expression progressively spreads from the dorsal side of the blastula margin towards ventral territories. This dynamic spreading of Fgf expression is concomitant with the disappearance of Bmp gene transcripts from dorsal cells and its restriction to the ventral part of the embryo. Transcripts for *fgf8* appear first at the dorsal margin (Fig. 1J) when we observe the beginning of Bmp gene clearance from the dorsal side of the embryo. Shortly afterwards, the expression of *fgf3* and *fgf24* becomes detectable in dorsal marginal blastomeres. As development proceeds, the expression domains of *fgf3* and *fgf24* progressively expand ventrally to form a DV gradient at the margin of the zebrafish blastula (Fig. 1K,L). The DV expression gradient of the Fgf-target gene *sprouty 4* (*spry4*) (Fürthauer et al., 2001) further suggests the existence of a DV gradient of Fgf activity at blastula stage (Fig. 1M-O). Taken together, our observations reveal a temporal coincidence between the progressive ventral restriction of Bmp gene expression and the ventralwards expansion of Fgf activity. The observation that the expression of both *fgf3* and *fgf24* occurs along a DV gradient at the margin of the zebrafish blastula suggests that these factors could be implicated in the establishment of DV patterning.

Fgf signalling affects pre-gastrula stage Bmp gene expression

In agreement with the hypothesis of an implication of Fgf signalling in the DV patterning of the zebrafish embryo, we found that overexpression of fgf3 (Fig. 2C; 57/57 embryos), fgf24 (Fig. 2D; 62/62 embryos) and fgf8 (Fig. 4P; 75/76 embryos) dorsalise the embryo, which adopts a characteristic elongated shape. This phenotype is similar to the one resulting from inactivation of Bmps (Kishimoto et al., 1997; Dick et al., 2000; Schmid et al., 2000) or overexpression of the Bmp-antagonist Nog1 (Fig. 2B; 38/39). This strongly suggests that Fgfs affect the DV patterning by interfering with Bmp signalling.

Because of the similarity between Bmp loss-of-function and Fgf gain-of-function phenotypes, and the temporal coincidence between expansion of Fgf gene expression and restriction of Bmp gene expression, we tested whether Fgf3/8/24 may affect early blastula stage Bmp gene expressions. Microinjection of *nog1* and *fgf8* RNAs leads to similar embryo morphology (Fig. 2B, Fig. 4P) but affects *bmp2b* differently (Fig. 2E-H). Although Nog1 does not affect early phases of Bmp gene expression (Fig. 2F), Fgf8 overexpression abolishes the blastula stage expression of *bmp2b* (Fig. 2G; 46/56 embryos), *bmp7* (Fig. 2J; 77/77 embryos) and *bmp4* (Fig. 2L; 53/57 embryos) in the ventral blastoderm. Whereas ectopic Fgf signalling inhibits Bmp gene expression in the ventral non-marginal blastoderm, it does not affect the expression of *bmp2b* and *bmp7* in the yolk syncytial layer (Fig. 2G,H,J) and the expression of *bmp4* at the dorsal margin (Fig. 2L). In addition, microinjection of *fgf3* (Fig. 2M, 34/53 embryos) or *fgf24* (Fig. 2N, 30/30 embryos) leads to inhibition of *bmp2b*, *bmp4* (not shown) and *bmp7* (not shown) expression at blastula stage.

A previous study revealed that an *fgf3*-mediated expansion of dorsolateral neurectodermal derivatives in the gastrula embryo depends on Chd function (Koshida et al., 2002). At early blastula, we found that Bmp gene expression is unaffected by Chd overexpression (Fig. 1H), suggesting that the inhibitory action of Fgfs on early Bmp gene expression is independent of Chd. To confirm this hypothesis, we showed that *fgf8* RNA injection in chordin morphants (mo-chd) or *chordino* mutants (not shown) causes severe dorsalisation (Fig. 2O, 79/87 embryos) and loss of early ventral *bmp2b* expression (Fig. 2P, 56/56 embryos) in the absence of Chd function.

The dorsalising activity of Fgf is mediated through the activation of the Ras/Map-kinase signal transduction cascade (Fürthauer et al., 2002). The downstream components of this signalling cascade are transcription factors belonging to the Ets family (Wasylyk et al., 1998). As targets of the Ras/Map-kinase signalling pathway, Ets proteins function as crucial nuclear integrators of this signalling cascade. Among the members of this family of transcription factors, two of them, Erm1 and Pea3 belong to the Fgf8 synexpression group and have been shown to respond to Fgf8, suggesting that they may be transcriptional mediators of Fgf signalling (Raible and Brand, 2001; Roehl and Nüsslein-Volhard, 2001). In accordance with this, localised overexpression of either *erm1* or *pea3* RNA causes ectopic expression of the Fgf target-gene *spry4* (Fig. 2Q, 51/58 embryos; Fig. 2S, 48/53 embryos compared with *spry4* expression in wild-type embryo, Fig. 1O). Misexpression of these transcription factors leads also to the local inhibition of *bmp2b* expression (Fig. 2R, 50/54 embryos; Fig. 2T, 57/62 embryos compared with *bmp2b* expression in wild-type embryo, Fig. 2E), demonstrating that these effectors of the Fgf signalling pathway are able to negatively regulate Bmp gene expression. Nevertheless, Erm1 and Pea3 are known to function as transcriptional activators (Sharrocks, 2001), suggesting that, although they are the mediators of the Fgf signalling pathway, they are not direct repressors of Bmp gene transcription.

Spry2 is a novel member of the Fgf8 synexpression group

If Fgfs are implicated in DV patterning, inactivation of their physiological antagonists should cause alterations of the DV organisation similar to Fgf overexpression. We found previously that Spry4 and Sef act as feedback inhibitors of Fgf signalling (Fürthauer et al., 2001; Fürthauer et al., 2002; Tsang et al., 2002). Inactivation of Sef or Spry4 results indeed in weakly dorsalised embryos. However, this phenotype is much

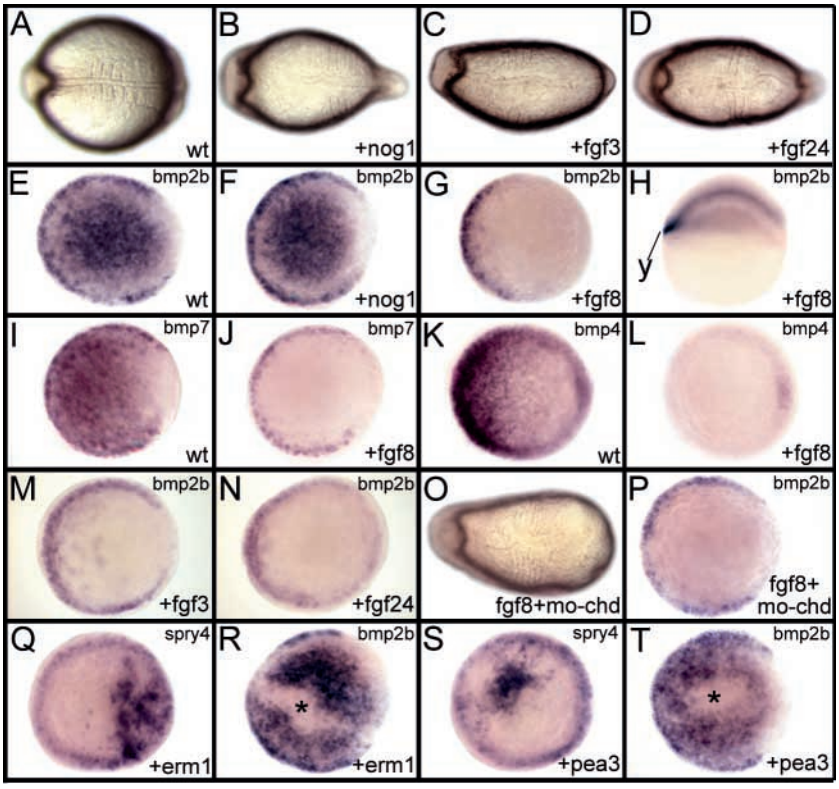

Fig. 2. Fgf signalling dorsalises the embryo by inhibiting Bmp gene expression. (A-D) Inhibition of Bmp signalling or Fgf overexpression cause dorsalisation phenotypes. Early segmentation stage morphology of wild-type (A), *nog1*-injected (B), *fgf3*-injected (C) and *fgf24*-injected (D) embryos. (E-N) Blastula stage Bmp gene expression is unaffected by inactivation of Bmp proteins, but lost following Fgf overexpression. All embryos are at 30% epiboly. Expression of *bmp2b* in wild-type (E), *nog1*-injected (F) and *fgf8*-injected (G,H) embryos (H is a lateral view of G, dorsal toward the right). (I,J) *bmp7* expression in wild-type (I) and *fgf8*-injected embryos (J). (K,L) *bmp4* expression in wild-type (K) and *fgf8*-injected embryos (L). (M,N) Loss of *bmp2b* expression following injection of *fgf3* (M) or *fgf24* (N). (O,P) *fgf8* overexpression dorsalises *chd*-depleted embryos. Characteristic dorsalised morphology (O) and loss of *bmp2b* expression at blastula stage (P) following co-injection of mo-*chd* and *fgf8* RNA. (Q,S) Ectopic expression of *spry4* expression after localised misexpression of RNA encoding Erm1 (Q) or Pea3 (S). (R,T) Local inhibition of *bmp2b* expression (asterisk) after localised misexpression of RNA encoding Erm1 (R) or Pea3 (T). (A-D,O) Dorsal views, anterior towards the left; (E-G,I-N,P-T) Animal pole views, dorsal towards the right.

less severe than the phenotype resulting from fgf8 overexpression (Fürthauer et al., 2001; Fürthauer et al., 2002).

We speculated that the weak dorsalisation of Spry4 or Sef-depleted embryos may be due to the existence of additional modulators of Fgf signalling. In accordance with this hypothesis, we isolated a second zebrafish Spry homologue, most similar to murine *spry2* (de Maximy et al., 1999; Tefft et al., 1999), referred to as zebrafish *spry2*. The distribution of *spry2* transcripts was analysed by in situ hybridisation and found to closely follow the expression of *fgf3*, *fgf8* and *fgf24* throughout embryonic development. *spry2* transcripts are maternally deposited in the egg. Although most maternally expressed RNAs are detected throughout the cytoplasm of early cleavage stage embryos, *spry2* transcripts display a strikingly different localisation. At the 32-cell stage highly localised, punctate distribution of *spry2* transcripts was revealed (Fig. 3A,B). After the activation of the zygotic genome, *spry2* transcripts start to be enriched at the margin of the blastula, showing overlap with expression domains of *fgf3*, *fgf8* and *fgf24* (Fig. 3C,D). Marginal expression of *spry2* persists as gastrulation proceeds (Fig. 3F). At midgastrulation, *spry2* expression becomes detectable in the presumptive midbrain/hindbrain region, which expresses *fgf8* (Fig. 3E,F); in the presumptive forebrain, which expresses *fgf3* (Fig. 3F) (Fürthauer et al., 2001); and in the axial mesendoderm expressing *fgf24* (Fig. 3F) (Draper et al., 2003). During segmentation, *spry2* is expressed, like *fgf8*, *spry4*, *sef*, *erm1* and *pea3*, in the telencephalon, midbrain-hindbrain region, heart primordia, somites and tail bud (Fig. 3G-L,U-X). Later, the cephalic expression of *spry2* resolves to the telencephalon, the dorsal diencephalon, the optic stalk and the midbrain-hindbrain boundary (Fig. 3N). *spry2* is further expressed in the anterior otic vesicle (like *fgf3* and *fgf8*) and in the branchial arch primordia (like *fgf3*; Fig. 3O,P). At 48 hpf, weak *spry2* expression is observed in the neurohypophysis, adjacent to the adenohypophyseal *fgf8* expression (Fig. 3Q,R). Complementary expression of these two genes is also observed in the pectoral fin, *fgf8* being expressed in the apical ectodermal ridge and *spry2* in the underlying mesenchyme (Fig. 3S,T). Expression profile analysis therefore identifies *spry2* as a novel member of the *fgf8* synexpression group. This also suggests that similarly to *spry4* (Fürthauer et al., 2001), *spry2* may be expressed in response to Fgf signalling.

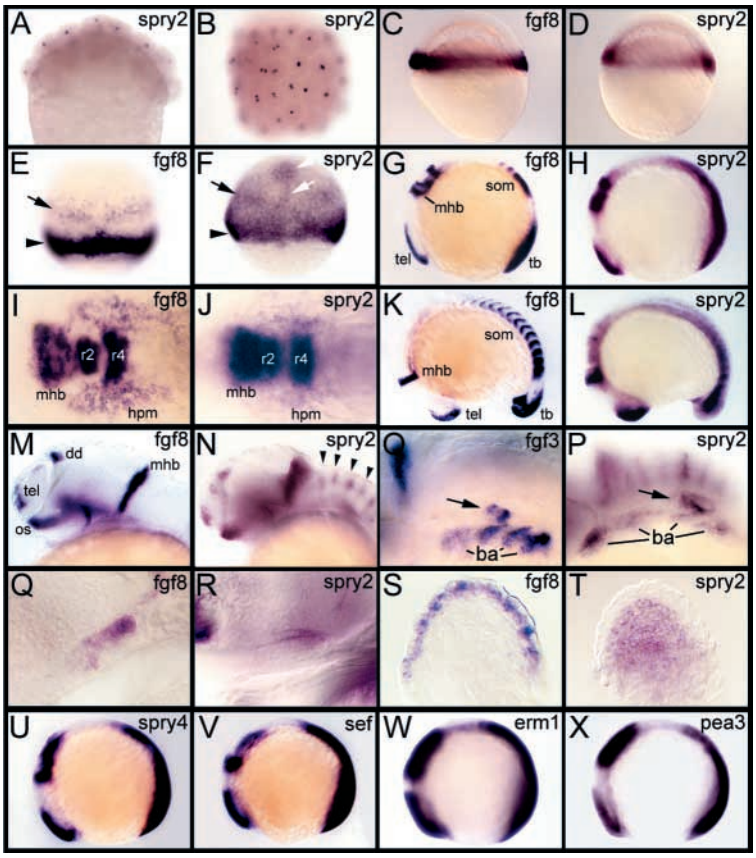

Fig. 3. *spry2* is a novel member of the *fgf8* synexpression group. Comparison of the expression pattern of *spry2* (A,B,D,F,H,J,L,N,P,R,T) with *fgf8* (C,E,G,I,K,M,Q,S), *fgf3* (O), *spry4* (U), *sef* (V), *erm1* (W) and *pea3* (X). (A,B) *spry2* transcripts in 32-cell stage embryos. (C,D) Late blastula stage expression of *fgf8* (C) and *spry2* (D). (E,F) Midgastrula stage expression of *fgf8* (E) and *spry2* (F); black arrowhead indicates margin; black arrow indicates presumptive midbrain-hindbrain region; white arrow indicates axial hypoblast; white arrowhead indicates presumptive forebrain. (G-J) Five-somite stage expression of *fgf8* (G,I) and *spry2* (H,J). (K,L) Expression of *fgf8* (K) and *spry2* (L) at the 16-somite stage. (M-P) Expression of *fgf8* (M), *fgf3* (O) and *spry2* (N,P) at 36 hours. Arrowheads indicate rhombomere boundaries; arrows indicate anterior otic vesicle. (Q-T) Expression of *fgf8* (Q,S) and *spry2* (R,T) at 48 hpf in the neurohypophysis (Q), adenohypophysis (R), the apical ectodermal ridge (S) and mesenchyme of the pectoral fin (T). (U,V) Five-somite stage expression of *spry4* (U), *sef* (V), *erm1* (W) and *pea3* (X). (A,C,D,G,H,K-R, U-X) Lateral views; (B) animal pole view; (I,J,S,T) dorsal views. Anterior is upwards in C-F and towards the left in G-X. ba, branchial arches; dd, dorsal diencephalon; hpm, heart primordia; mhb, midbrain hindbrain boundary; os, optic stalk; r2/r4, rhombomeres 2 and 4; som, somites; tb, tail bud; tel, telencephalon.

According to this hypothesis, inhibition of Fgf signalling by microinjection of RNA encoding a dominant-negative variant of zebrafish Fgfr1 (dn-fgfr1) causes a severe reduction or complete loss of *spry2* expression (Fig. 4B, 56/56 embryos). In *fgf8/acerebellar* mutant embryos, expression of *spry2* is reduced in telencephalon, somites and tail bud, and completely lost at the level of the midbrain-hindbrain boundary (MHB) (Fig. 4E,F). Importantly, other MHB-markers are still expressed at this stage in *ace* mutant embryos (Reifers et al., 1998), indicating that loss of *spry2* is not due to lack of the MHB region, but is rather a direct consequence of impaired Fgf8 activity.

Conversely, overexpression of fgf8 RNA, either throughout the embryo or at the animal pole (which is far from its endogenous marginal expression) induces ectopic *spry2* expression (Fig. 4C, 36/36 embryos; Fig. 4D, 58/58 embryos). Taken together, these findings establish *spry2* as a novel Fgf target gene.

Sprouty2 is necessary to inhibit dorsalising Fgf signals

Because *spry2* is a target of Fgf, and as *Drosophila* Spry antagonises Fgf signalling (Hacohen et al., 1998), we tested

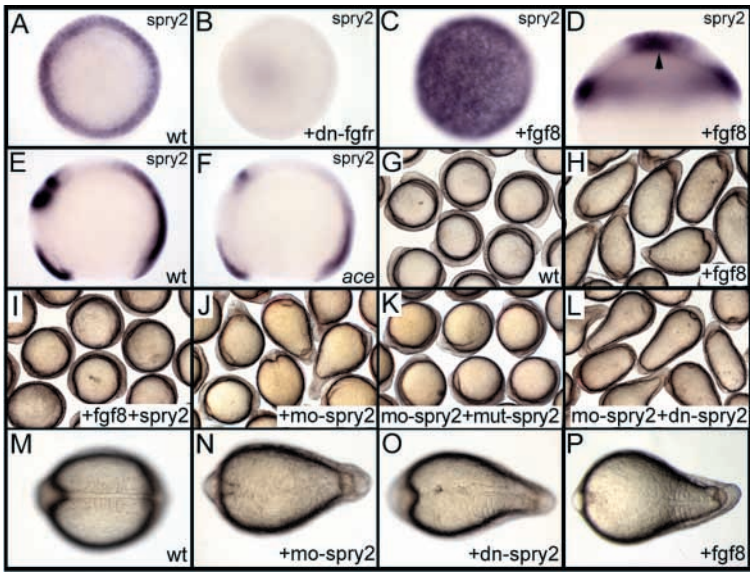

Fig. 4. Spry2 is a feedback inhibitor of Fgf signalling. (A) Wild-type expression of *spry2*. (B) Inhibition of Fgf signalling by injection of RNA encoding dn-fgfr abolishes *spry2* expression. (C) Ubiquitous or (D) localised (arrowhead) overexpression of *fgf8* RNA induces ectopic *spry*2 expression. (F) *spry2* expression is reduced in *fgf8/ace* mutant embryos compared with wild-type siblings (E). Compared with wild-type (G) embryos injected with *fgf8* RNA (H) display dorsalised phenotypes. (I) Co-injection of *fgf8* with *spry2* RNA rescues embryos. (J,O,L) Injection of (J) mo-spry2 or (O) dn-spry2, or (L) co-injection of both induce dorsalised phenotypes. Compare with injection of *fgf8* RNA (H,P). (K) Dorsalisation is abolished by co-injection of mut-spry2. (A-D) Late blastula stage, (E-P) early segmentation stage. (A-C) Animal pole views; (D-L) lateral views; (M-P) dorsal views. (E,F,M-P) Anterior is towards the left.

whether *spry2* is able to inhibit Fgf activity. Although injection of fgf8 alone causes a severe dorsalisation, resulting in the elongation of embryos at the beginning of somitogenesis (Fig. 4H, 62/69), co-injection of *fgf8* and *spry2* causes embryos to revert to wild-type morphology (Fig. 4I, 69/69 embryos). This observation shows that *spry2* is able to antagonise dorsalising Fgf signalling.

If Spry2 is important for the modulation of dorsalising Fgf signals under physiological conditions, then inhibition of Spry2 function should alter the DV patterning of the embryo. In accordance with this hypothesis, 31.8% (*n*=110) of the embryos injected with a morpholino directed against *spry2* display a moderately dorsalised phenotype at early segmentation stages (Fig. 4J) and 38.2% embryos a severely dorsalised phenotype (Fig. 4J,N), similar to *fgf8* overexpression phenotype (Fig. 4P) or genetic loss of Bmp2b or Bmp7 function (Kishimoto et al., 1997; Dick et al., 2000; Schmid et al., 2000). The specificity of the morpholino was demonstrated by the rescue of this dorsalisation (Fig. 4K, *n*=64) after co-injection of a full-length spry2 RNA in which the sequence recognised by the morpholino has been mutated (mut-spry2, see Materials and methods).

The effect of Spry2 on DV patterning was confirmed by injection of an RNA encoding a dominant-negative form (Hanafusa et al., 2002) of the mouse *spry2* (Fig. 4O; 45.6% moderately and 25.6% strongly dorsalised, *n=90*). Co-injection of RNA encoding this dominant-negative form with spry2 morpholino further enhances the penetrance of the dorsalised phenotype (Fig. 4L; 18.1% weakly and 73.6% severely dorsalised embryos, *n*=144).

In accordance with the hypothesis that Fgf signalling affects the DV patterning through the inhibition of Bmp gene expression, inhibition of spry2 function by co-injection of spry2 morpholino and DN-Spry2 RNA, which results in an increase of Fgf activity, causes a reduction of *bmp2b* expression in the ventral blastoderm (Fig. 5A-D, 47/47). Inactivation of Spry2 causes an expansion of the expression domain of a marker of dorsal ectoderm (anterior neurectoderm) cyp26a (Fig. 5E,F; 50/50 embryos) and the concomitant reduction of ventral ectoderm (epidermis) revealed by *foxi1* expression (Fig. 5I,J; 40/44 embryos). The dorsalising effect of *spry2* loss of function can be abolished by co-injection of

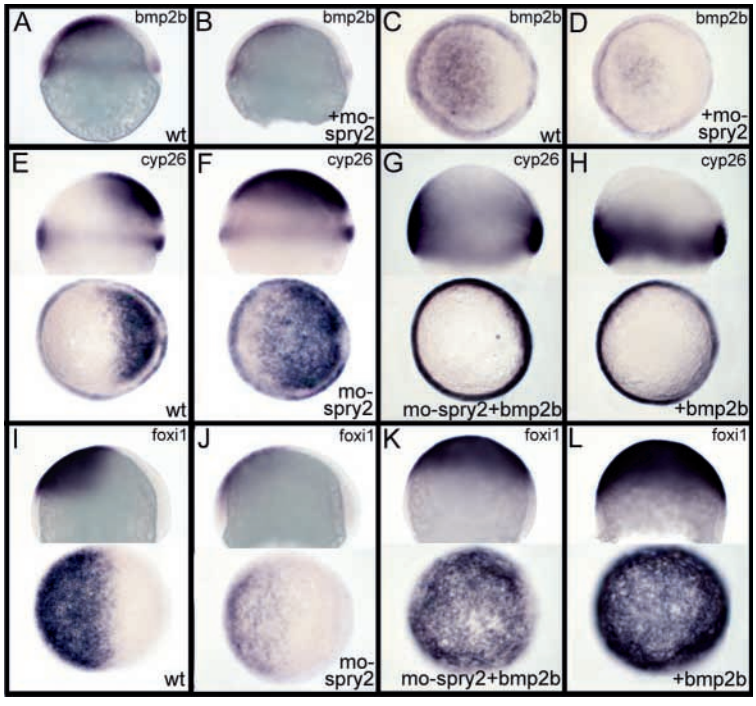

Fig. 5. Inhibition of *spry2* alters dorsoventral patterning. (A-D) Embryos co-injected with mo-spry2 and dn-spry2 display severely reduced *bmp2b* expression. (E-L) Increased Bmp signalling levels cause alterations of DV patterning in spry2 loss-of-function experiments. (F) Injection of mo-spry2 causes an expansion of *cyp26a*-expressing dorsal anterior neurectoderm, compared with (E) wild type (wt), which is abolished by co-injection of *bmp2b* (G) similar to inhibition of neural fate after injection of *bmp2b* alone (H). (I,J) mo-spry2-injected embryos display a reduction of the *foxi1*-expressing ventral epidermis. Co-injection of *bmp2b* rescues and even enlarges the epidermal territory (K) similar to a single *bmp2b* injection (L). (A-D) Shield stage; (E-L) midgastrula stage. (A-D,E-L, top) Lateral views. (E-L, bottom) Animal pole views; all embryos are dorsal towards the right.

bmp2b: *cyp26a* disappears from dorsal ectoderm (Fig. 5G; 13 reduced, 35 abolished, *n*=48), while *foxi1* expression is rescued and even enlarged (Fig. 5K; 15 wild-type, 23 enlarged, *n*=38).

Taken together, our results show that the upregulation of endogenous Fgf signalling levels following inhibition of Spry2 causes a severe reduction of Bmp gene expression and concomitant alterations of DV patterning (summarised in Fig. 9D).

Inhibition of Fgf upregulates Bmp signalling

We then examined whether Fgf signalling is required for the dorsal clearance of *bmp2b* expression at blastula stages. Inhibition of Fgf signalling by injection of RNA encoding the Fgf-antagonist Spry2 results in expanded *bmp2b* expression (Fig. 6B, 63/69). A similar expansion was observed following injection of dn-fgfr1 (Fig. 6C, 66/80), injection of a dominant-negative variant of the signal transducer Ras (not shown) or treatment with the pharmacological Fgfr antagonist SU5402 (Mohammadi et al., 1997) (Fig. 6D, 26/26). Taken together, these experiments demonstrate that Fgf signalling is required in vivo to restrict early *bmp2b* expression to the ventral side of the embryo (Fig. 9E). This expansion of the bmp2b expression territory results in an increase of Bmp signalling activity as revealed by a strong spreading of *ved* expression (a Bmp-responsive gene) (Shimizu et al., 2002) towards the dorsal side of the embryo (Fig. 6E,F; 29/40 embryos).

As a result of increased Bmp activity, Fgf-depleted embryos display pronounced alterations of their DV patterning in both mesodermal and ectodermal germ layers: expression of the dorsal mesodermal markers *goosecoid* (Fig. 6G,H; 34/37) and *sonic hedgehog* (Fig. 6I,J; 48/49) are severely reduced or completely lost. Conversely *draculin*, a marker of the ventral hypoblast corresponding to the presumptive blood territory (Herbomel et al., 1999) expands dorsally in embryos injected with dominant-negative Ras (dn-ras, Fig. 6K,L; 40/48) or dn-fgfr1 (not shown). In the ectodermal germ layer, inhibition of Fgf signalling causes a severe reduction of expression of the pan-neural marker *zic2* (Fig. 6M-P; 43/44).

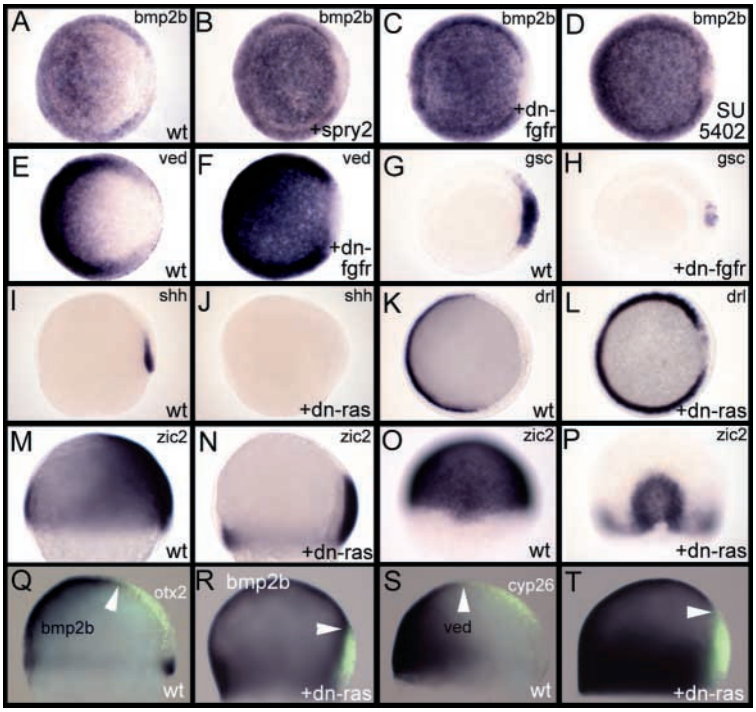

Fig. 6. Fgf-mediated restriction of Bmp gene expression is essential for dorsoventral patterning. *bmp2*b expression in wild type (A), or following overexpression of *spry2* (B), injection of RNA encoding a dn-fgfr (C) or treatment with SU5402 (D). (E,F) Following dn-fgfr injection, the expression of the Bmp target gene *ved* expands dorsally. (G-J) Inhibition of Fgf signalling reduces or abolishes the expression of dorsal mesodermal markers *goosecoid* (*gsc*, G,H) and *sonic hedgehog* (*shh*, I,J). (K,L) Conversely, the expression territory of *draculin* (drl), a marker of the ventral hypoblast, is expanded compared with wild type. (M-P) Compared with wild type (M,O) dn-ras injection causes a severe reduction of the expression domain of the pan-neural marker *zic2* (N,P). (Q,R) Two-colour in situ hybridisation with *bmp2b* (blue) and the anterior neural marker *otx2* (green) or (S,T) with *ved* (blue) and *cyp26a* (green). Arrowheads (Q-T) indicate the border between the gene expression domains. (A-H) Shield stage, (I-T) 70% epiboly. (A-H) Animal pole views; (I,J,M,N,Q-T) lateral views; (K,L) optical cross-section at the level of the margin; (O,P) dorsal views. (A-N,Q-T) Dorsal towards the right.

Because inhibition of Bmp signalling is known to be required for the specification of dorsal ectoderm (neural fates), reduction of the neural plate may be due to the expansion of *bmp2b* expression. To test this hypothesis, we carried out two colour in situ hybridisation to visualise simultaneously *bmp2b* expression and the limits of the neural plate, outlined by the forebrain marker *otx2* (Fig. 6Q,R). We found that both in wild-type and in Fgf-depleted embryos, the expression domains of *bmp2b* and *otx2* abut each other (Fig. 6Q,R, arrowhead). When Fgf signalling is inhibited, *bmp2b* expression expands dorsally and *otx2* becomes restricted to the small residual *bmp2b*-free zone next to the dorsal margin (Fig. 6R). Similarly, the expression of the anterior neural marker *cyp26a* abuts the expression of the Bmp-target *ved* both in wild-type and in dn-ras-injected embryos (Fig. 6S,T). Again, *cyp26a* expression becomes confined to the residual ved-free domain of dn-ras injected embryos (Fig. 6T). These observations strongly suggest that the reduction of the neurectoderm (dorsal ectoderm) observed in Fgf-signalling deficient embryos is due to increased Bmp signalling levels.

To confirm this hypothesis, we tested the capacity of the Bmp antagonist Nog1 to rescue ectodermal patterning in Fgf signalling-deficient embryos. The transcription factor *foxi1* is expressed in the presumptive epidermis (Fig. 7A), a tissue that requires high Bmp signalling levels (Wilson and Hemmati-Brivanlou, 1995). After inhibition of Fgf signalling, the epidermal territory expands dorsally (Fig. 7B, 43/44 embryos). Co-injection of RNA encoding Nog1 saves this DV patterning defect and even completely abolishes *foxi1* expression (Fig. 7C; dn-ras injected embryos 42/45 embryos), similar to the Nog1 overexpression phenotype (Fig. 7D, 43/43).

Previous analysis has shown that Fgf signalling is involved in the establishment of the anteroposterior patterning of the neural plate (Kudoh et al., 2002). We therefore analysed the expression of markers of either the anterior or the posterior neurectoderm in Fgf-depleted embryos. The expression of

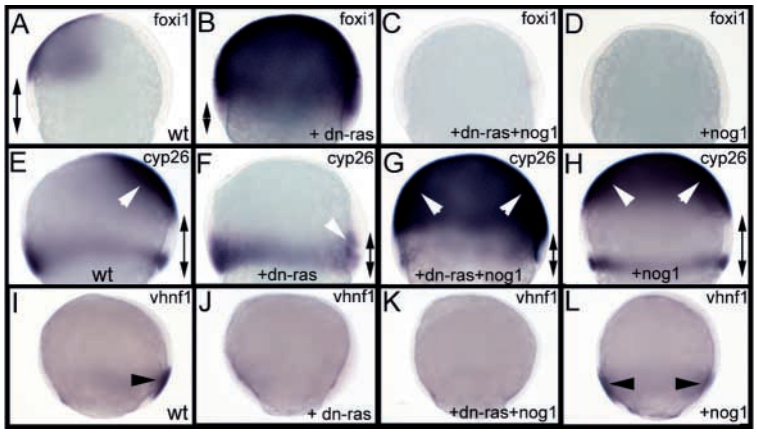

Fig. 7. Inhibition of Bmp signalling restores the ectodermal DV patterning in Fgf-depleted embryos. Analysis of the DV patterning of the ectoderm with *foxi1* (A-D), *cyp26a* (E-H) and *vhnf1* (I-L). Black arrows indicate the space between the blastoderm margin and the ectodermal gene expressions. (B) Microinjection of dn-ras causes an expansion of *foxi1* expression, (F) a reduction of the neural expression domain of *cyp26a* (white arrowhead) and (J) a complete loss of *vhnf1*. (C) Co-injection of *nog1* with dn-ras abolishes epidermal cell fates, (G) expands the anterior neurectoderm but (K) does not rescue *vhnf1* expression. (D) *nog1* injection abolishes *foxi1* expression, while the neural expression domains of *cyp26* (H) and *vhnf1* (L) expand ventrally. (A-H) 75% epiboly stage; (I-L) 85% epiboly stage. All embryos are shown in lateral view, dorsal towards the right.

cyp26a in the anterior neurectoderm is severely reduced and shifted marginally (Fig. 7F, 50/51). Co-injection of the Bmp-antagonist Nog1 restores and even expands the presumptive anterior neurectoderm (Fig. 7G; 48/49), showing that reduction of anterior neurectoderm in Fgf-depleted embryos is due to excessive Bmp signalling. However, the co-injection of Nog1 does not prevent the marginal shift of *cyp26a* expression (Fig. 7G). In addition, the analysis of *vhnf1*, a marker of posterior hindbrain and anterior spinal chord (Fig. 7I) reveals that its expression is decreased or lost in Fgf-depleted embryos (Fig. 7J, 55/55). This result is in good agreement with the requirement of Fgf for the specification of posterior neural fates. However, although injection of Nog1 into wild-type embryos expands *vhnf1* expression ventrally because of its dorsalising activity (Fig. 7L, 41/45), Nog1 injection fails to rescue *vhnf1* expression in dn-ras-injected embryos (Fig. 7K). Therefore, the inhibition of Bmp activity is able to rescue the ventralisation phenotype but not the lack of posterior neural fates induced by Fgf loss of function. This shows that Fgf signalling affects only the DV but not the AP patterning of the neurectoderm through the regulation of Bmp signalling.

Taken together, we show that enhanced Bmp signalling in Fgf-depleted embryos causes major alterations in the DV patterning of both mesodermal and ectodermal germ layers. Our results demonstrate that the Fgf-mediated ventralwards restriction of Bmp gene expression at blastula stage is essential for the establishment of the DV axis of the early zebrafish embryo (see Fig. 9F).

Chordin and Fgf8 cooperate to ensure proper DV patterning

Although overexpression of Fgf3/8/24 causes pronounced alterations of DV patterning, inactivation of these factors has only very minor (Reifers et al., 1998) or no effects on DV patterning (Draper et al., 2003) (M.F., C.T. and B.T., unpublished). We show here that modulation of Bmp activity is ensured both by Fgf-mediated inhibition of Bmp expression and by Bmp-binding antagonists. This suggests that the contribution of individual Fgfs to DV patterning may be masked by the predominant role of *chd*, a major Bmp-binding antagonist. To test this hypothesis, we analysed whether inactivation of Fgf8 affects DV patterning in the context of Chd-deficient embryos. In a first experiment, the phenotype of embryos injected with a morpholino against *chd* was compared with the phenotypes of embryos in which the function of both *chd* and *fgf8* was abolished through morpholino injection. Injection of Fgf8 morpholino has no effect on DV patterning (Fig. 8B). Following injection of Chd morpholino, of the embryos display a moderate (79.3%, *n*=87, Fig. 8C) or a severe expansion of the ventral haematopoietic mesoderm (17.3%, *n*=87). This expansion of ventral haematopoietic mesoderm is considerably enhanced by co-injection of a Fgf8 morpholino with 82.4% of the embryos displaying a severe expansion of the ventral mesoderm (Fig. 8D; *n*=125).

In a second experiment, Fgf8 morpholino was injected into homozygous mutant *chordino* (*din*) embryos. Simultaneous loss of Fgf8 and Chd function enhances the expansion of the ventroposterior mesoderm in 100% of the injected homozygous *din* mutants (*n*=27). In addition, as expected for an increase of Bmp protein signalling, 92.6% of the injected mutants display a reduction of the head compared to uninjected *din* homozygous embryos (Fig. 8E-H).

In a third experiment, Chd morpholino was injected into *ace/fgf8* mutant embryos. *ace* homozygous mutant embryos are easily recognisable because of the lack of *engrailed3* (*en3*) expression at the midbrain-hindbrain boundary (Fig. 8I,K). These embryos were probed for the expression of the *hemoglobin* (*hem*) gene. Injection of Chd morpholino causes a moderate enlargement of the haematopoietic territory and a severe enlargement in 86.7% (Fig. 8J) and 13.3%, respectively, of the injected *ace* heterozygotes (*n*=30). By contrast, injection into *ace* homozygous mutants results into 90% of the embryos displaying a severe expansion of the ventral mesoderm (Fig. 8L, *n*=30).

In order to assess whether the observed morphological changes result from early DV patterning alterations, we analysed the expression of the dorsal ectodermal marker *cyp26a* and the ventral mesodermal marker *draculin*. Following Chd morpholino injection, neural *cyp26a* expression is moderately reduced in 64.9% (Fig. 8O) and severely reduced in 35.1% of the embryos (*n*=37). Co-injection of Chd and Fgf8 morpholinos leads to a severe reduction or a nearly complete loss of neural *cyp26a* (77.1%; *n*=35). Similarly, combined inhibition of chd and fgf8 causes a more pronounced dorsal expansion of *draculin* (Fig. 8T; mean angular extent 313°, *n*=39) than the loss of Chd function alone (Fig. 8S, mean angular extent 279°, *n*=45). Taken together, our results show that Fgf8 and Chd act redundantly to ensure the proper

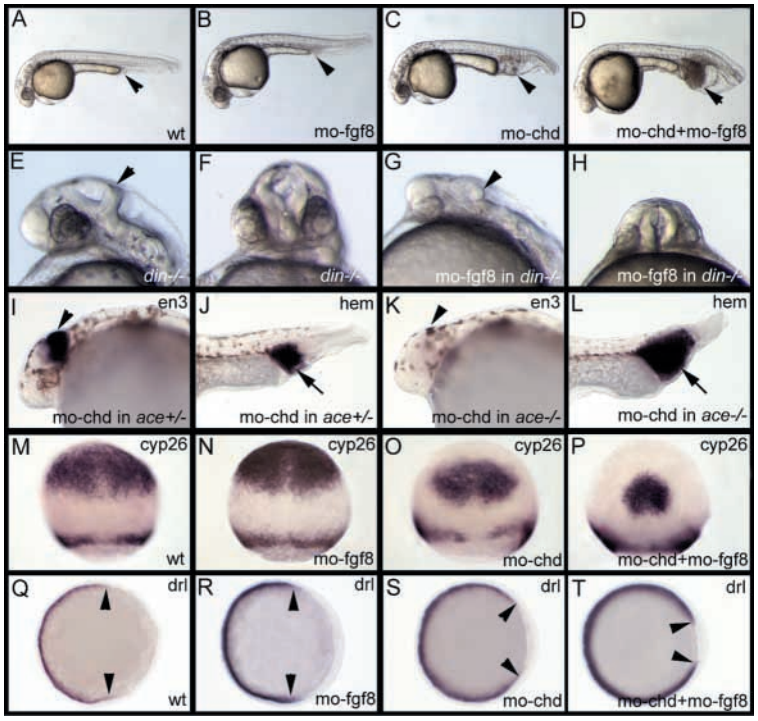

Fig. 8. Fgf8 and chordin interact genetically. (A-D) Expansion of the ventral mesoderm (arrowheads) in embryos injected with chordin morpholino (mo-chd) (C) is enhanced by co-injection of mo-fgf8 (D) when compared with injection of mo-fgf alone (B) or with wild type (A). (E-H) Injection of mo-fgf8 causes a reduction of head size in *chordino* (*din*) mutant embryos. mo-fgf8 injection leads to loss of the cerebellum (arrowheads). (I-L) *ace/fgf8* mutation enhances the expansion of the ventral hematopoietic mesoderm induced by mo-chd injection. (I,K) Loss of en3 expression (arrowheads) identifies *ace* homozygous mutants (K). (J,L) Expression of *hemoglobin* (*hem*) (arrows) after injection of mo-chd in heterozygous $ace^{+/-}$ embryo (J) and in homozygous $ace^{-/-}$ mutant (L). (M,N) Injection of mo-fgf8 does not affect *cyp26a* expression (N) compared with wild type (M). (O,P) The reduction of neural *cyp26a* expression caused by mo-chd (O) is further enhanced by co-injection of mo-fgf8 (P). (Q,R) Injection of mo-fgf8 does not affect *drl* expression (R) compared with wild type (Q). (S,T) The dorsal expansion of the expression of ventral mesodermal marker *draculin* (*drl*) caused by mo-chd (S) is further enhanced by co-injection of mo-fgf8 (T). Arrowheads in S,T indicate the dorsal limit of the *drl* expression domain. (A-E,G,I-L) Lateral views, anterior towards the left. (F,H) Frontal views, dorsal towards the top. (M-P) Dorsal views, anterior upwards. (Q-T) Optical sections through the margin, dorsal towards the right. (A-L) 30 hpf, (M-T) 75% epiboly.

modulation of Bmp activity that is required to establish DV patterning.

Discussion

Fgf signalling controls the DV patterning of the zebrafish

Previous genetic studies showed that the DV patterning of the zebrafish is dependent on morphogenetic Bmp signalling (Kishimoto et al., 1997; Dick et al., 2000; Schmid et al., 2000). The generation of the Bmp activity gradient involves the inhibition of ventrally secreted Bmps by their dorsally released antagonists Chd and Nog (Piccolo et al., 1996; Zimmerman et al., 1996). The observation that Nog or Chd do not affect blastula stage Bmp gene expression indicates that different mechanisms are involved in the regulation of Bmp protein activity and in the regulation of Bmp gene expression (Fig. 9B). We have shown that the progressive restriction of Bmp RNAs to the ventral side is mediated through Fgf signalling that is initiated on the dorsal side of the embryo and progressively spreads to more lateral and ventral domains (Fig. 9A,C).

The decrease of Bmp gene expression and the concomitant dorsalisation of embryos depleted for the Fgf-signalling antagonist *spry2* shows clearly that Fgfs, when signalling from their endogenous expression territories, can affect the DV patterning of the zebrafish (Fig. 9D). Most importantly, inhibition of Fgf signalling through the use of dominant-negative Fgf receptors, the endogenous Fgf-signalling antagonist *spry2* or pharmacological antagonist SU5402 causes a dorsalwards expansion of Bmp gene expression and a concomitant expansion of ventral cell fates (Fig. 9E).

Taken together, our work shows that in addition to the well-known interaction between Bmps and Bmp antagonists, the establishment of the DV axis of the zebrafish embryo requires the ventral restriction of Bmp genes expression at blastula stage, a process controlled by the Fgf signalling pathway (Fig. 9A).

Contribution of individual Fgfs to DV patterning

Despite their dorsalising activities, inactivation of individual Fgfs fail to show any effect on DV patterning. This may be due to a functional redundancy between Fgf family members. Accordingly, complete inhibition of Fgf signalling results in pronounced DV patterning alterations, demonstrating that the Fgf pathway is required for the control of this process.

A functional redundancy is also observed amongst Bmp-binding antagonists. Loss of Chd function results in embryos displaying an incomplete ventralisation, which is enhanced by the simultaneous loss of *ogon* activity (Miller-Bertoglio et al., 1999b).

Chd and Fgfs affect DV patterning through different mechanisms: Chd abolishes Bmp signalling by binding Bmp proteins (Fig. 9B). By contrast, Fgf overexpression abolishes Bmp gene expression (Fig. 9C). In both cases, the ultimate outcome is a loss of Bmp signalling, which results in an expansion of dorsal cell fate concomitant with loss of ventral cell fates.

Chd has a very strong inhibitory effect on Bmp that makes it difficult to detect the effect of a weak decrease in the dorsal inhibition of Bmp gene expression by loss-of-function of a single Fgf. In accordance with this hypothesis, in Chd loss-of-function mutant, inactivation of Fgf8 enhances the ventralisation phenotype providing therefore genetic evidence for the implication of Fgf signalling in early DV patterning.

Fgf signalling and neural induction

Although some studies suggest that inhibition of ectodermal Bmp signalling is sufficient for the acquisition of neural identity, experiments carried out in the *Xenopus* and chicken

suggest a requirement for Fgf signalling for the early acquisition of neural competence (Streit and Stern, 1999). According to this second view, Fgf signalling would be required for an early phase of neural induction during the blastula period that cannot be achieved by the Bmp antagonists Nog and Chd (Streit et al., 2000). Our study shows that, in the zebrafish, Fgf signalling affects DV patterning already at blastula stage. We do however find that the requirement for the early action of Fgfs can be bypassed if Bmp signalling is inhibited by microinjection of Nog. Our observations are therefore similar to those of Wilson and co-workers (Wilson et al., 2000), which suggest that neural cell fate specification starts at blastula stages with an Fgf-mediated inhibition of Bmp expression in the domain of the prospective neural plate.

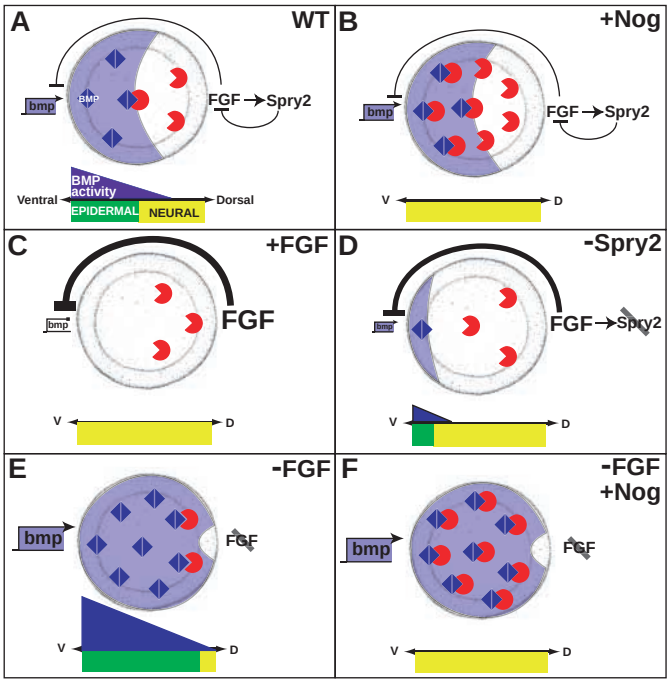

Fig. 9. DV patterning of the early zebrafish embryo. (A) Two levels of regulation of the morphogenetic Bmp activity gradient. First, the action of Bmp proteins (dark blue squares) is inhibited by chordin and noggin (red), which bind Bmps and prevent them from interacting with their receptors. Second, Fgfs affect the DV patterning by restricting the domain of Bmp gene expression (light blue). Fgf signals are modulated by feedback-inhibitors such as Spry2. The combined regulation of Bmp gene expression and Bmp protein activity results in the generation of a Bmp activity gradient (dark blue) that determines the identity of cells along the DV axis. Cells that experience high levels of Bmp activity will adopt a ventral (e.g. epidermal, green; ventral, V) fate, cells that experience a low Bmp activity a more dorsal (e.g. anterior neurectodermal, yellow; dorsal, D) fate. (B) Noggin overexpression does not affect early Bmp gene expression but abolishes Bmp activity by complexing all available Bmp molecules. As a result, the ventral epidermis is lost, to the benefit of the dorsal neurectoderm. (C) Fgf overexpression abolishes Bmp gene expression and therefore also Bmp activity. As for Noggin-injected embryos, the ventral epidermis is lost at the expense of the dorsal neurectoderm. (D) Loss of function of the Fgf-signalling antagonist *spry2* causes an upregulation of endogenous Fgf signalling and a decrease of the Bmp transcription domain. The dorsal neurectoderm is expanded at the expense of the epidermis. (E) Following inhibition of Fgf signalling, Bmp gene transcripts are expressed throughout the embryo with the exception of the dorsalmost marginal blastomeres. As a consequence, the ventral epidermis expands while the dorsal neurectoderm is severely reduced in size. (F) Overexpression of Noggin in Fgf-depleted embryos inactivates Bmp proteins. Consequently, all cells adopt a dorsal neurectodermal fate.

Previous work in *Xenopus* and zebrafish (Dosch et al., 1997; Barth et al., 1999) revealed that Bmp signalling specifies DV identity in both mesoderm and ectoderm. In addition, our past studies have shown that formation of the neurectoderm does not depend on the presence of mesoderm (Thisse and Thisse, 1999; Thisse et al., 2000). Consequently, neural induction should not be viewed as a distinct process but as one aspect of the DV patterning: the definition of dorsal ectodermal territories.

We show here that Fgf, through the control of Bmp activity, affects DV patterning in both mesoderm and ectoderm. Therefore, rather than considering our results as an evidence for a role of Fgf signalling in neural induction, they provide evidence that Fgf-mediated regulation of Bmp expression is essential for the DV patterning of both mesodermal and ectodermal derivatives.

Multiple effects of Fgf signalling

Fgfs are known for their implication in posterior development (Kudoh et al., 2002; Cox and Hemmati-Brivanlou, 1995; Kengaku and Okamoto, 1995; Griffin et al., 1995), mesoderm induction and maintenance (Schulte-Merker and Smith, 1995; Isaacs et al., 1994) (for a review, see Yasuo and Lemaire, 2001). We show in the present study that Fgf signalling does also affect the DV axis of the early zebrafish embryo. Early in development, inhibition of Fgf signalling does not cause the loss of mesoderm but a dorsal expansion of ventral mesodermal fates concomitant with a progressive loss of dorsal mesoderm. In addition, overexpression of Fgf, either by RNA injection or by inhibition of its feedback antagonist *spry2*, affects the DV patterning of both mesoderm and ectoderm but does not result in an increase of mesoderm. Therefore, it appears clearly that at blastula stage Fgf controls cell identity along the DV axis rather than the establishment of the mesodermal germ layer. This territory is lost later in development, during gastrulation. This process is likely independent of Bmp signalling as loss of Bmp gene function (in *bmp2b*/*swr*) (Kishimoto et al., 1997) or in *bmp7*/*snh* (Schmid et al., 2000) affects ventral mesoderm formation but does not prevent formation of axial or paraxial mesoderm. In addition, Bmp gain of function (through Bmp RNA injections) affects DV patterning but not mesoderm induction.

In addition to its function in DV patterning and mesoderm formation, the Fgf signalling pathway is also involved in posterior development of the embryo. In particular, at gastrulation, inhibition of Fgf activity results in loss of posterior neurectoderm. We show here that this effect is independent of Bmp signalling. The inhibition of Bmp activity through overexpression of Nog1 is able to rescue DV patterning defect but fails to rescue the posterior neurectoderm. In addition the loss of posterior neurectoderm is also independent of mesoderm formation as posterior neurectoderm can be formed in absence of both endoderm and mesoderm (Thisse and Thisse, 1999; Thisse et al., 2000). Finally, we observe that the sensitivity of Fgf8 and Fgf3 for DV and AP patterning are not identical (not

shown). At low doses (injection of 0.2 pg RNA), Fgf8 affects DV patterning with little effect on AP patterning. At the opposite, low dose (injection of 1 pg RNA) of Fgf3 posteriorises the neurectoderm, whereas little or no effect is observed on DV patterning. At higher dose (1 pg Fgf8 RNA and 5 pg Fgf3 RNA) both DV and AP patterning are affected by these two ligands. Two Fgf receptors, Fgfr1 and Fgfr4 are expressed at blastula stage (Thisse et al., 1995) (M.F., C.T. and B.T., unpublished). One explanation for the pleiotropy of Fgf signalling may reside in the difference in affinity of Fgf ligand for the different Fgf receptors that may act through the stimulation of different downstream signalling pathways (Klint and Claesson-Welsh, 1999).

Altogether, these observations suggest that the three functions of Fgfs during early zebrafish development, mesoderm formation, AP and DV patterning are distinct. We show in this report that Fgfs, which signal through the Ras-MAP kinase pathway, regulate the DV patterning at the level of Bmp gene expression. However, the effect on AP patterning may involve interactions between Fgfs and other posteriorising factors, such as Wnt8. Finally, the effect on mesoderm formation is likely to imply interactions with Nodal signalling.

Understanding how the action of a single signalling pathway can contribute to mesoderm formation, DV and AP patterning, and the nature of the interaction between Fgfs and Wnt or Nodal signalling pathways will be the major challenges for future studies.

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