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The entire zebrafish blastula-gastrula margin acts as an organizer dependent on the ratio of Nodal to BMP activity

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Formation of the vertebrate embryo is known to depend on the activity of organizing centers. The dorsal Spemann organizer is the source of growth factor antagonists that participate in the creation of signaling gradients. In various species, the existence of head, trunk and trunk-tail inducers has been proposed to explain the formation of different parts of the embryo along the anteroposterior (A/P) axis. In zebrafish, two organizing centers have been described, the dorsal and tail organizers, located at the dorsal and ventral gastrula margins, respectively. Here, we report that organizer functions are executed not only by the dorsal and ventral margins, but also by all parts of the blastula-gastrula margin. The position of different marginal territories along the dorsoventral axis defines the A/P nature of the structures they are able to organize. At the molecular level, we show that this organizing activity results from the simultaneous activation of BMP and Nodal signaling pathways. Furthermore, the A/P character of the organized structures is not defined by absolute levels but instead by the ratio of BMP and Nodal activities. Rather than resulting from the activity of discrete centers, organization of the zebrafish embryo depends on the activity of the entire margin acting as a continuous and global organizer that is established by a gradual ventral-to-dorsal modulation of the ratio of marginal BMP to Nodal activity.

KEY WORDS: Zebrafish, Organizer, Margin, BMP, Nodal, Blastula, Gastrula

INTRODUCTION

Spemann and Mangold showed that the organization of the vertebrate embryo depends on the activity of a dorsal center, referred to as the Spemann organizer (Spemann and Mangold, 1924). They defined the organizer activity as the ability of the dorsal blastopore lip of an amphibian gastrula to induce a secondary axis, containing both donor and host cells, when transplanted into the ventral side of a host embryo. Equivalents of the Spemann organizer have been identified in other vertebrates. In teleosts, it is located at the dorsal gastrula margin and corresponds to the embryonic shield. When grafted into the ventral margin of a host embryo, secondary head and trunk structures are formed (see Fig. 1A,B) (Oppenheimer, 1936; Saude et al., 2000). Molecular analyses performed during the past 15 years have shown that the Spemann organizer acts by secreting BMP antagonists such as Chordin, Noggin, Follistatin or Follistatin-like 2 (DeRobertis, 2006; Dal-Pra et al., 2006), as well as Wnt antagonists such as Frzb and Dickkopf (Niehrs, 2004; DeRobertis, 2006), which are responsible for the establishment of a ventral-to-dorsal BMP and Wnt–β-catenin gradient of activity (Harland and Gerhart, 1997; Niehrs, 2001; Niehrs, 2004). Local inhibition of BMP on the ventral side of the zebrafish embryo, where this morphogen activity is maximal, leads to the formation of a secondary axis; however, this twinned embryo is incomplete (Fürthauer et al., 1999) as it is devoid of axial structures (prechordal plate, notochord and floor plate). By contrast, the local misexpression at the ventral margin of two secreted inhibitors, such as Noggin 1 for BMP and Frzb for Wnt8, results in the formation of a second embryonic axis with a fully developed head and trunk (see Fig. S1A-C in the supplementary material) (Glinka et al., 1997; Yasuo and Lemaire, 2001). However, a similar misexpression at the animal pole never induces formation of a secondary axis. This reveals that the full organization of the embryo requires more than the inhibition of BMP and Wnt activities. In particular, the Nodal signaling pathway has been implicated in axis formation and patterning, suggesting its involvement in organizing activity (Jones et al., 1995; Lustig et al., 1996; Feldman et al., 1998; Thisse et al., 2000). Accordingly, overexpression of the Nodal signaling pathway at the ventral blastula-gastrula margin results in the formation of complete secondary embryonic axes (see Fig. S1D in the supplementary material) (Lustig et al., 1996; Renucci et al., 1996; Erter et al., 1998; Sampath et al., 1998) similar to those induced in response to the double inhibition of Wnt and BMP or after grafting the dorsal margin in a ventral-marginal position.

Evidence of organizer subdivision in amphibians was first proposed by Mangold (Mangold, 1933) (reviewed by Stern, 2005). He found that transplantation of early gastrula lips into the blastocoel of host embryos results in the formation of secondary heads, whereas transplantation of late gastrula lips induces secondary trunk structures. Signals for head induction reside in the anterior visceral endoderm of mice, whereas, in birds and amphibia, they are located in the prechordal plate mesendoderm (reviewed by Harland and Gerhart 1997; Pera and Kessel, 1997; Beddington and Robertson, 1999; Knoetgen et al., 1999; Kimura et al., 2000; Kinder et al., 2001). The identification of Cerberus in *Xenopus* revealed that the formation of the cephalic territory results from the triple inhibition of BMP, Nodal and Wnt (Piccolo et al., 1999). Conversely, trunk-tail inducing properties can be generated in *Xenopus* after overexpression of BMP inhibitors (reviewed by Harland and Gerhart, 1997; Niehrs, 2004). In zebrafish, we have previously demonstrated that the ventral blastula-gastrula margin acts as an organizing center independently of the dorsal organizer and that this part of the margin is responsible for the formation of the zebrafish tail through the triple stimulation of Nodal, BMP and Wnt8 (Agathon et al., 2003).

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In the present study, we establish that, in zebrafish, cell populations lying at the blastula-gastrula margin, other than those corresponding to the dorsal and ventral organizers, are capable of recruiting and engaging naïve cells from the animal pole into developmental programs that lead to the formation of the tissues and organs of the three germ layers. By performing animal pole grafts of various domains of the early gastrula margin, we found that all grafted territories display organizing activity. Therefore, rather than resulting from the activity of discrete centers, the formation of the embryo is controlled by the entire blastula-gastrula margin, which acts as a global organizer. At the molecular level, we found that this global organizer activity results from the interaction between BMP and Nodal factors. Finally, we established that it is the ratio of stimulation of these two signaling pathways that defines the anteroposterior (A/P) nature of the structures organized by the different regions of the blastula-gastrula margin.

MATERIALS AND METHODS

Cell transplantation and injection into animal pole blastomeres

Cell transplantation and RNA injection were performed as previously described (Agathon et al., 2003). Transplantations were performed by grafting early gastrula (shield stage) marginal cells into the animal pole of a host blastula (dome stage, i.e. 30% epiboly). Co-injection of BMP and Nodal was performed using *bmp2b* and *nodal-related 2* (*ndr2*, *cyclops*) RNAs transcribed from a pCS2+ plasmid (digested with *Not*I) using an SP6 RNA polymerase kit (Ambion). Results similar to those reported for *bmp2b* and *ndr2* were observed with other BMP (*Bmp4*, *Bmp7*) and Nodal (*nodal-related 1*, *squint*) ligands, except that the amount of RNA injected had to be varied from gene to gene to obtain structures with the same A/P identity.

For the *nodal* injection, the amount of RNA used (3 pg) corresponds to the highest dose generating ectopic structures while still allowing the development of the embryo for 2 days after fertilization. Injection of higher doses kills the embryo at the gastrula stage.

In situ hybridization

Wholemount in situ hybridizations were performed as described previously (Thisse and Thisse, 2008). Information for the synthesis of probes for *krox20* (*egr2a/b* – Zebrafish Information Network), *cadherin 17* (*cdh17*), *homeobox C12b, antivin* (*lefty1*), *even-skipped-like*, *ventrally expressed dharma/bozozok antagonist* (*ved*), *homeobox C13b*, *engrailed 2*, *solute carrier family 4 anion exchanger member 2*, *fatty acid binding protein 10 liver basic (fabp10)*, *retinoid x receptor* γ and *starmaker* are available from the Zebrafish Information Network. The *solute carrier family 12 anion exchanger member 3* (*slc12a3*) corresponds to the IMAGE clone 7037010, and its antisense RNA synthesis was performed by digestion with *Eco*RI using the T7 RNA polymerase. For *si:dkey-70p6.3p*, a pseudo-gene described in the Vega database, the template is a PCR amplification (GenBank number DX503783) of one exon using the primers CCCAGAAGAGAATGAACCAG and GGATCCATTAACCCTCACTAAAGGGAACTTGTGTCCACCAATTT-CAG, and the probe is synthesized using T3 RNA polymerase. For *shh*, the construct in a pBluescript vector was digested with *Hin*dIII, and the probe was synthesized using T7 RNA polymerase. For *ndr1*, the construct in a pBluescript vector was digested with *Eco*RI and the probe was synthesized using T7 RNA polymerase.

RESULTS

The lateral blastula-gastrula margin displays organizing properties

In a previous study focusing on the role of the margin in the organization of the zebrafish embryo, we showed that the ventral margin has a tail organizer activity (Fig. 1A,D) (Agathon et al., 2003). Conversely, the zebrafish dorsal margin, equivalent to the Spemann organizer in the frog, when transplanted to the animal pole of a blastula, results in the formation of axial structures in a territory normally fated to become the telencephalon and the eye (Fig. 1A,C) (Agathon et al., 2003). At the blastula stage, this dorsal marginal territory expresses a set of genes involved in the repression of Wnt signaling (Niehrs et al., 2001; Thisse et al., 2001; Seiliez et al., 2006). Such repression is required to prevent the posteriorization of the anterior neural plate by Wnt8. Therefore, although the ventral margin is involved in the organization of the tail, the dorsal margin organizes the axial structures and is required for the formation of the most anterior part of the embryo. In order to determine whether other regions of the zebrafish lateral margin also have organizer properties, we grafted portions of the lateral early gastrula margin from a donor embryo to the animal pole of a host embryo (Fig. 1E). The grafted embryos developed ectopic structures extending from the anterior tip of the primary axis that

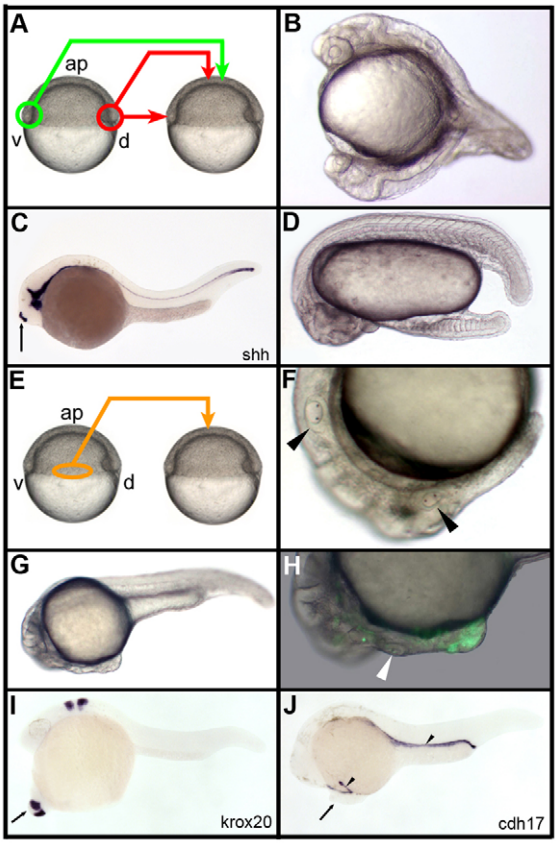

Fig. 1. Organizing properties of the early gastrula margin. (**A**) Dorsal (red) and ventral (green) marginal grafts of the early gastrula embryo. (**B**) Transplantation from the dorsal to ventral margin organizes secondary head and trunk. (**C**) A graft of the dorsal margin at the animal pole induces a short stretch of notochord labeled with the sonic hedgehog *shh* probe (arrow). (**D**) A graft of the ventral margin at the animal pole results in the formation of a secondary tail. (**E-H**) Grafts of the lateral margin (orange, E) organize partial secondary axes containing anterior trunk and posterior head structures (arrowheads in F, otic vesicles in endogenous and secondary axes) that extend from the endogenous head (G) and contain both GFP-labeled cells from the donor, as well as unlabeled cells from the host (H; arrowhead, secondary otic vesicle). (**I**,**J**) In situ hybridizations using *krox20* and *cdh17* RNA probes. Arrow in I,J, secondary axes; arrowhead in J, pronephric ducts; ap, animal pole. B-D,F-J, lateral view, anterior to the left, dorsal to the top; A,E, lateral view, anterior to the top.

displayed properties identical to the posterior head and trunk regions [e.g. induction of a second set of otic vesicles (Fig. 1F)]. These structures comprised fluorescently labeled cells from the donor, as well as unlabeled cells from the host that had been recruited from the animal pole to form these anterior ectopic secondary axes (Fig. 1G,H). In situ hybridizations with *krox20* and *cadherin 17* RNA probes revealed the presence of ectopic hindbrain rhombomeres 3 and 5 (Fig. 1I) and secondary pronephric ducts (Fig. 1J), respectively. Various transplanted pieces of the lateral margin were thus able to recruit and organize host animal pole cells, fated to become the telencephalon and eyes, into well-differentiated embryonic structures with posterior head and trunk identity. Therefore, in accordance with the definition of an organizer, as originally established by Spemann and Mangold (Spemann and Mangold, 1924), the zebrafish lateral margin acts as an organizer of the posterior head and trunk.

The blastula-gastrula margin acts as a spatially continuous organizer

Based on this last observation, it appears that three organizing centers – the dorsal organizer, the tail organizer and the trunk-posterior head organizer, which lies at the lateral blastula margin – control the organization of the embryo. These organizer activities were found to be independent of each other, as grafting various marginal domains at the animal pole results in the formation of structures corresponding to different parts of the body along the A/P axis. In order to characterize more precisely the position and extent of these organizing centers, we performed a series of transplantations using marginal tissue derived from different positions along the dorsoventral axis at the animal pole of a host embryo (Fig. 2A). All grafts led to the formation of ectopic structures that branched anteriorly to the endogenous head (Fig. 2B-I) and comprised both donor cells and cells recruited from the animal pole region of the host (see Fig. S2 in the supplementary material). The A/P nature of the structures generated was probed by in situ hybridization using *hoxC12b*, a marker for the tail, or *krox20*, a marker for the posterior head. As described in Fig. 2, various ectopic structures were obtained and classified into four groups: the 'tail' class, characterized by positive expression for *hoxC12b* and negative expression for *krox20* (Fig. 2B,C); the 'trunk' class, with no expression of *hoxC12b* but expression of *krox20* in two rhombomeres (Fig. 2D,E); the 'posterior head' class, characterized by short structures that remain close to the endogenous head and fail to express either *hoxC12b* or *krox20*, or else express *krox20* in only one rhombomere (Fig. 2F,G); and, the 'axial structures' class, characterized by the presence of a morphologically recognizable notochord, the absence of expression of *hoxC12b* and the expression of *krox20* in a few cells (Fig. 2H,I).

The frequency of the different types of ectopic structures was analyzed (Fig. 2J). Grafts of the ventral margin resulted predominantly in the formation of ectopic tails, with some embryos displaying ectopic trunk or posterior head structures. The frequency of ectopic tails decreased dramatically when, instead of ventral margin, lateral margin (90°, LM) was grafted to the animal pole. In this case, most of the induced structures share trunk and posterior head identity. Similar observations were made for grafts of the dorsolateral margin (45°, DLM), with fewer embryos belonging to the 'trunk' class and an increased number of embryos developing axial structures. Finally, grafts of the dorsal margin resulted mainly in the formation of ectopic axial structures, with a few embryos belonging to the 'posterior head' class.

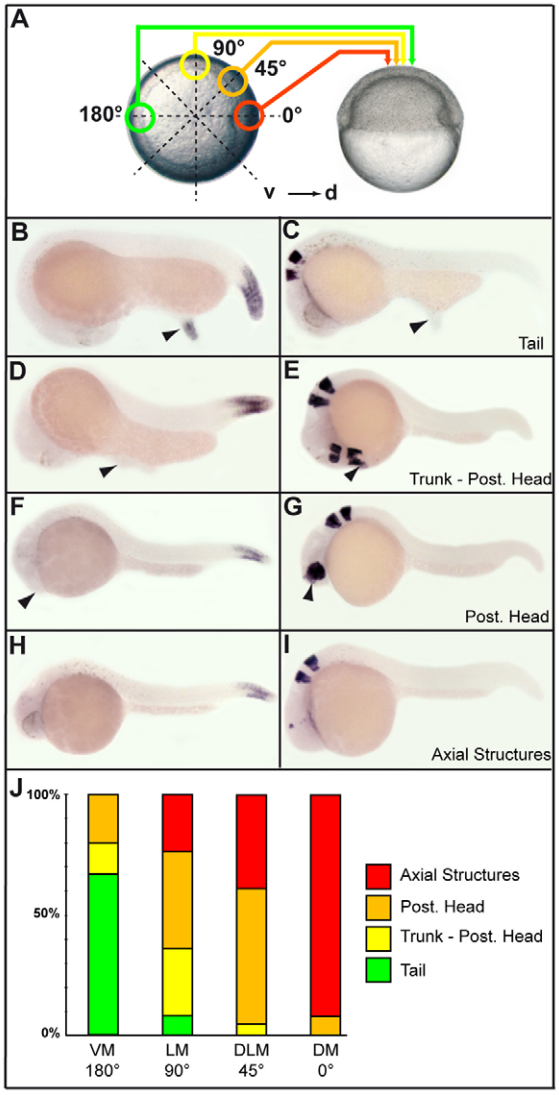

Fig. 2. Continuous organizing properties of the early gastrula margin. (**A**) Various gastrula marginal grafts performed at the animal pole of host embryos. Donor embryo, left, animal pole view; host embryo, right, lateral view. 0°, dorsal margin (DM); 45°, dorsolateral margin (DLM); 90°, lateral margin (LM); 180°, ventral margin (VM). (**B-I**) In situ hybridizations for *hoxC12b* (B,D,F,H) and for *krox20* (C,E,G,I) on embryos displaying secondary tails (B,C), a trunk-posterior head (D,E), a posterior head (F,G) and axial structures (H,I). Arrowheads indicate secondary axes. (**J**) The frequency of the different structures induced by grafts of the various pieces of margin. Number (*n*) of embryos analyzed: DM,15; DLM,18; LM, 24; VM, 15. B-I, lateral view, anterior to the left, dorsal to the top.

In summary, we observed a progressive change in the class of structures generated as we changed the dorsoventral origin of the graft tissue. The more ventral the source of the graft, the more posterior was the nature of the structures developed. This shows a co-linearity between the dorsoventral position of blastula-gastrula margin cells that are grafted and the portion of the embryo along the A/P axis that they are able to organize.

These observations argue against the existence of three different and discrete organizing centers and strongly support the idea that the entire zebrafish margin acts as a continuous and global organizer.

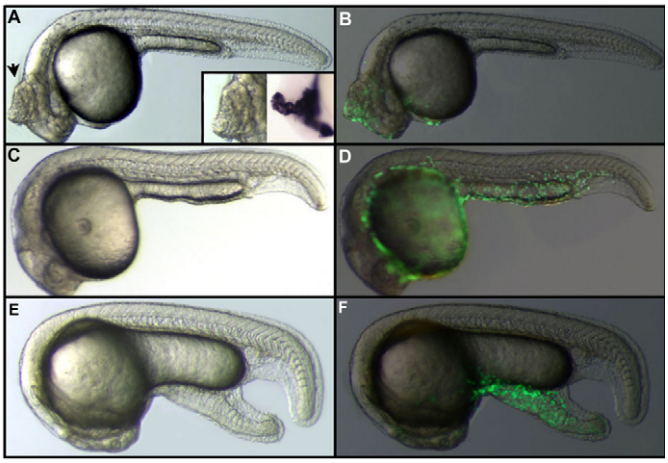

Fig. 3. Effect of Nodal or BMP on animal pole blastomeres. (**A**) Injection of *nodal* RNA in an animal pole blastomere at the 128- to 256-cell stage results in the formation of ectopic axial structures (arrow) that express *shh*, a marker of the notochord and floor plate (inset). (**B**) Localization of the clone derived from the injected blastomere visualized by fluorescence resulting from the translation of *gfp* RNA co-injected with *nodal* RNA. (**C**) Injection of *bmp4* RNA in an animal pole blastomere at the 128- to 256-cell stage has no effect on the morphology of the embryo. (**D**) Cells derived from the injected blastomere are all located in the epidermis. (**E**) Injection of *nodal* and *bmp4* RNAs in an animal pole blastomere at the 128- to 256-cell stage results in the formation of an ectopic tail. (**F**) Localization of the clone derived from the injected blastomere visualized by fluorescence resulting from the translation of *gfp* RNAs co-injected with *nodal* and *bmp4* RNAs.

Dual stimulation of the Nodal and BMP signaling pathways mimics the organizing properties of the blastula-gastrula margin

In a previous study, we implicated the Nodal, BMP and Wnt signaling pathways in the organizing activities of both the dorsal and ventral zebrafish margin (Agathon et al., 2003). In particular, stimulation of animal pole cells by injection of Nodal (*ndr2*) and *gfp* mRNAs at the 128-cell stage resulted in the formation of ectopic axial structures (Fig. 3A,B) and mimicked the effect of a graft of the dorsal margin to the animal pole of a host embryo. By contrast, injection of BMP (*bmp2b*) RNA into an animal pole blastomere had no effect on the morphology of the embryo (Fig. 3C,D); the clone derived from the injected blastomere populated only the ventral epidermis. Nevertheless, the double stimulation of animal pole cells by BMP and Nodal resulted in the formation of an ectopic tail, devoid of axial structures, growing from the animal pole; this ectopic tail contained both labeled cells derived from the injected blastomere and unlabeled cells recruited from the animal pole of the embryo (Fig. 3E,F) (Agathon et al., 2003). This double stimulation was sufficient to mimic the graft of ventral marginal cells to the animal pole of a host blastula.

Interestingly, Nodal-related genes *ndr1* and *ndr2* are expressed all around the margin at the blastula stage (Feldman et al., 1998; Rebagliati et al., 1998), with *ndr2* being expressed at a higher level on the dorsal side. In addition, expression of *antivin* (Thisse et al., 1999), an early target of Nodal, demonstrates that the activity of this pathway is not restricted to the dorsal margin but is instead constant along the dorsoventral axis of the blastula margin. By contrast, the activity of BMP decreases in a ventral-to-dorsal manner, as revealed by the expression pattern of *bmp2b* at the late blastula stage (Nikaido et al., 1997; Fürthauer et al., 2004) or by the expression of homeo box protein Ved, a target of the BMP pathway (Shimizu et al., 2002). These observations, combined with the data from our grafting experiments, suggest that the continuous organizing activity of the blastula-gastrula margin could depend on a variation in BMP activity in the presence of a rather constant level of Nodal. We probed this hypothesis by injecting a constant amount of *nodal* RNA, corresponding to the minimum amount required to mimic the graft of the dorsal margin to the animal pole, together with a range of different amounts of *bmp* RNA into an animal pole blastomere at the 128-cell stage. After co-injection, embryos were allowed to develop until 24-72 hours post-fertilization (hpf) and were subsequently analyzed morphologically, as well as for the expression of specific molecular markers. In all conditions used, we observed the formation of ectopic structures developing at the animal pole and extending from the endogenous head region. Embryos were sorted based on the morphology of the ectopic structures and then analyzed by in situ hybridization using a set of markers specific for different parts of the embryo and for different germ layers. We defined four different classes of induced structures. First, the 'posterior structures or tail' class (Fig. 4Ba-h), characterized by the expression of *hox13b*, a specific marker of the tail, as well as the expression of *slc12a3*, a marker of the most-posterior part of the pronephric ducts. Second, the 'posterior trunk' class (Fig. 4Ca-h), characterized by structures labeled with the *slc4a2a* probe specific for the anterior pronephric duct, *fabp10* (a marker of the liver) and *si:dkey-70p6.3p* (a marker of pectoral fins at 72 hpf) but failing to express the tail/posterior markers. Third, the 'anterior trunk and posterior head' class, characterized by an ectopic anterior spinal cord as revealed by *rxrg*, by the presence of a second set of otic vesicles labeled by *starmaker*, and duplication of the midbrain-hindbrain boundary labeled by *engrailed 2* (Fig. 4Da-h). Some embryos belonging to this third class exhibited labeling for the anterior pronephric ducts marker, whereas the 'posterior trunk' class comprised some embryos with a weak labeling for *rxrg*. In these cases, embryos are assigned to either the 'posterior trunk' or 'anterior trunk/posterior head' classes in relation to the posterior extension of the induced structure (a shorter ectopic structure being classified into the 'anterior trunk/posterior head' class, whereas longer secondary axes were classified into the 'posterior trunk' class). Finally, the fourth, 'axial structures' class, was characterized by embryos displaying a morphologically visible notochord growing from the head. In situ hybridization revealed that these embryos do not express any of the previously described molecular markers (Fig. 4Ea-h). The frequency of embryos belonging to these different classes was determined for each experimental condition (Fig. 4F). We observed a range of phenotypes for each given amount of *bmp* RNA injected (0 to 75 pg) together with a constant amount of *nodal* RNA (3 pg). Formation of anterior structures (labeled for *starmaker* or *engrailed 2*) was observed mainly after co-injection of *nodal* with 3 pg of *bmp* RNA, whereas the frequency of ectopic trunk organs, such as the liver or pectoral fins, was higher after co-injection with 15 pg of *bmp* RNA. Finally, the frequency of formation of posterior structures increased progressively with co-injection of increasing amounts of *bmp* and was maximal at the highest amount (75 pg) of *bmp* RNA injected.

This gradual increase in the posterior character of the ectopic structures generated upon stimulation of the animal pole with Nodal and BMP is in good agreement with the gradual activity of BMP at the blastula-gastrula margin. The shape, extent and A/P identity of the structures formed in response to an animal pole injection of

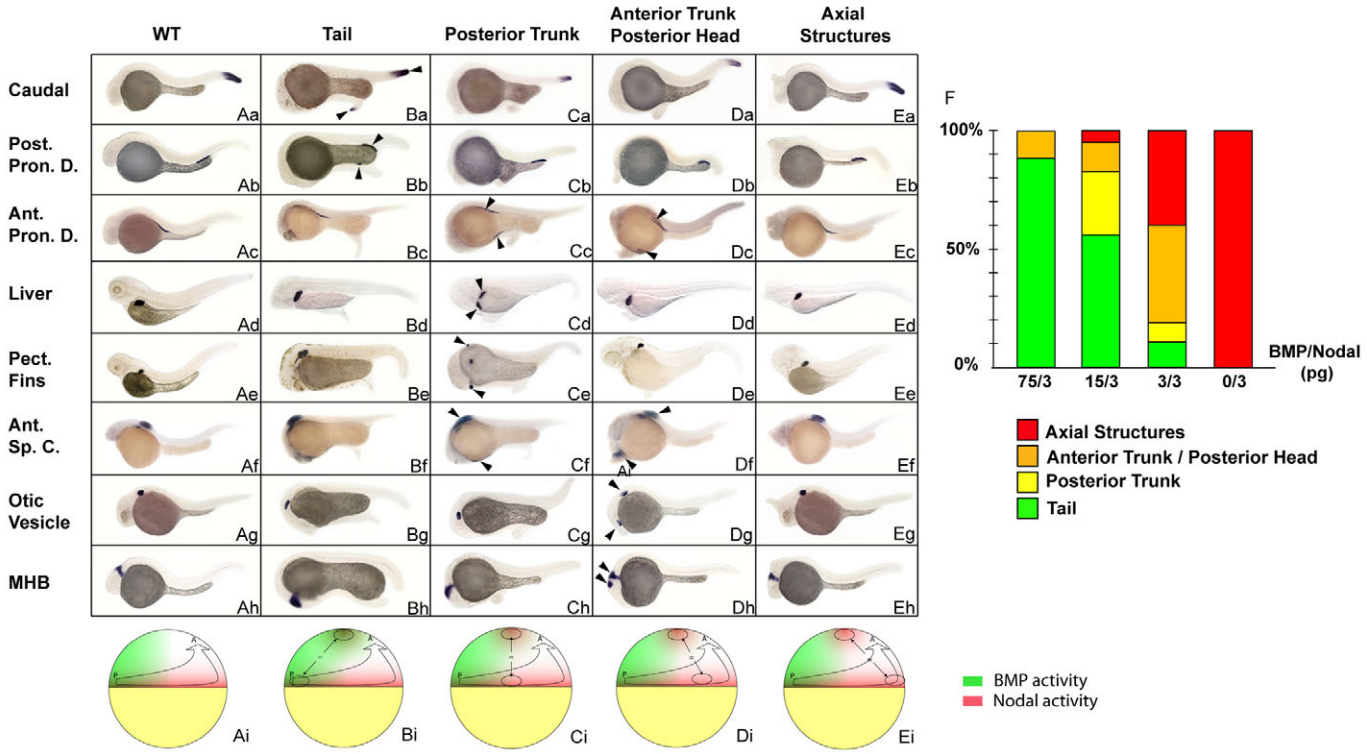

Fig. 4. Simultaneous stimulation of Nodal and BMP activities recapitulates the organizing activities of the different marginal domains. (**Aa-Eh**) In situ hybridization with markers of caudal structures (*hoxC13b*, first row), posterior pronephric ducts (Post. Pron. D., *slc12a3*, second row), anterior pronephric ducts (Ant. Pron. D., *slc4a2*, third row), liver (*fabp10*, fourth row), pectoral fins (Pect. Fins, *si:dkey-70p6.3p*, fifth row), anterior spinal cord (Ant. Sp. C., *rxrγ*, sixth row), otic vesicles (*starmaker*, seventh row) and the midbrain-hindbrain boundary (MHB, *engrailed 2*, eighth row) on embryos co-injected with *nodal* and a range of *bmp* RNAs in an animal pole blastomere at the 128-cell stage and sorted based on the morphology of the induced secondary structures, as indicated at the top. WT embryo (Aa-h), embryo displaying a secondary tail (Ba-h), posterior trunk (Ca-h), anterior trunk/posterior head (Da-h) and axial structures (Ea-h). (Ai-Ei) Schematic representation of embryos at late blastula stage, dorsal to the right, depicting the type of marginal grafts at the animal pole of a host blastula that result in the formation of the structures indicated above the figure. Green, the ventral domain corresponding to high levels of BMP; red, the activity of Nodal at the blastula margin. (**F**) The frequency of the different classes generated after injection of various dilutions of *bmp* and *nodal* RNAs. Green, tail; orange, posterior trunk; yellow, anterior trunk/posterior head; red, notochord; WT, wild type; arrowheads, endogenous and ectopic structures formed. Lateral view. For each condition, at least 120 embryos were injected in two independent experiments.

different combinations of *bmp* and *nodal* RNAs were identical to those of the structures organized at the animal pole as a result of grafts of different regions of the blastula-gastrula margin (Fig. 4Ai-Ei). Tail structures were generated for grafts of the ventral-most margin or by injection of high amounts of *bmp* for a constant amount of *nodal* (Fig. 4Bi). Posterior trunk or anterior trunk/posterior head structures were observed for grafts of the lateral or dorsolateral margin, respectively, or by co-injection of 3 pg of *nodal* RNA with a progressively decreasing amount of *bmp* RNA (Fig. 4Ci-Di). This clearly shows that the stimulation of animal pole cells by various combinations of Nodal and BMP efficiently reproduces the organizing activities identified for the different parts of the margin.

Anteroposterior identity depends on the ratio of BMP/Nodal activities

The previous experiment showed that, for a given constant level of stimulation by Nodal, a range of stimulation by BMP can organize tissues and organs corresponding to the different parts of the embryo along the A/P axis. Therefore, the organizing activity could depend strictly on the absolute value of BMP activity, which is known to be tightly regulated (De Robertis, 2006). Alternatively, the organizing activity generated by the double stimulation of BMP and Nodal might depend not on the absolute level of activation of these two signaling pathways, but on their relative level of activity. We probed these hypotheses by injecting an animal pole blastomere with different concentrations of *nodal* and *bmp* RNAs but maintaining a constant *nodal*/*bmp* ratio. We assumed that the level of activity of each signaling pathway is directly correlated to the amount of RNA encoding their respective ligands. We began with the condition that generates an ectopic tail (injection of 3 pg *nodal* RNA together with 75 pg *bmp* RNA). We then injected a range of dilutions of this RNA mix, up to a 1:16 dilution (which corresponds to a final amount of 0.19 pg *nodal* and 4.7 pg *bmp* RNA). For all dilutions tested, the majority of injected embryos developed ectopic tail structures (70-86%; Fig. 5A). Therefore, a simple variation in the amount of *bmp* RNA is not sufficient to change the A/P identity of the structures organized at the animal pole when the *nodal*/*bmp* RNA ratio is maintained constant. For a 1/25 ratio, posterior structures are generated preferentially. We performed the same analysis for a 1/1 ratio, starting with a mix of 3 pg *nodal* and 3 pg *bmp* RNAs, then diluting this mix of RNAs up to a 1:16 dilution. These various injections resulted in the formation of ectopic structures that display mainly trunk/posterior head identity (50-81%; Fig. 5B). This second set of experiments using a 1/1 ratio also supports our hypothesis that

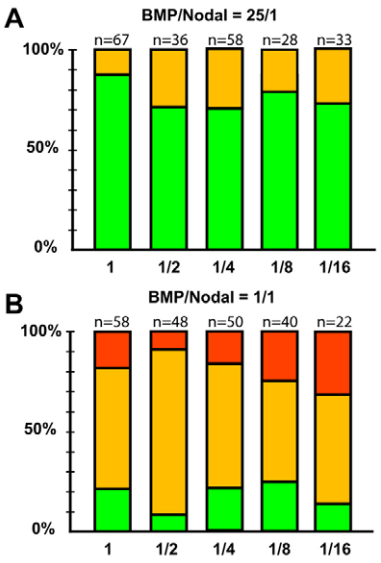

Fig. 5. The A/P character of the organizing activity is controlled by the BMP/Nodal ratio. (**A**,**B**) The percentage of embryos displaying a secondary tail (green), trunk and posterior head (orange) or axial structures (red) after injection of various dilutions of *nodal* and *bmp* RNAs into an animal pole blastomere at the 128-cell stage. Serial dilutions of *nodal* and *bmp* RNAs were used, keeping the *bmp*/*nodal* ratio constant. A 25/1 ratio of *bmp*/*nodal* RNAs mainly induces tails (A). The same result is obtained for all dilutions of RNA mixes injected (dilution up to 1:16). When a 1/1 ratio is used, the embryos preferentially develop ectopic intermediate structures (B, trunk and posterior head). The same result is obtained for serial dilutions of this RNA mix (dilution up to 1:16). *n*, number of embryos injected.

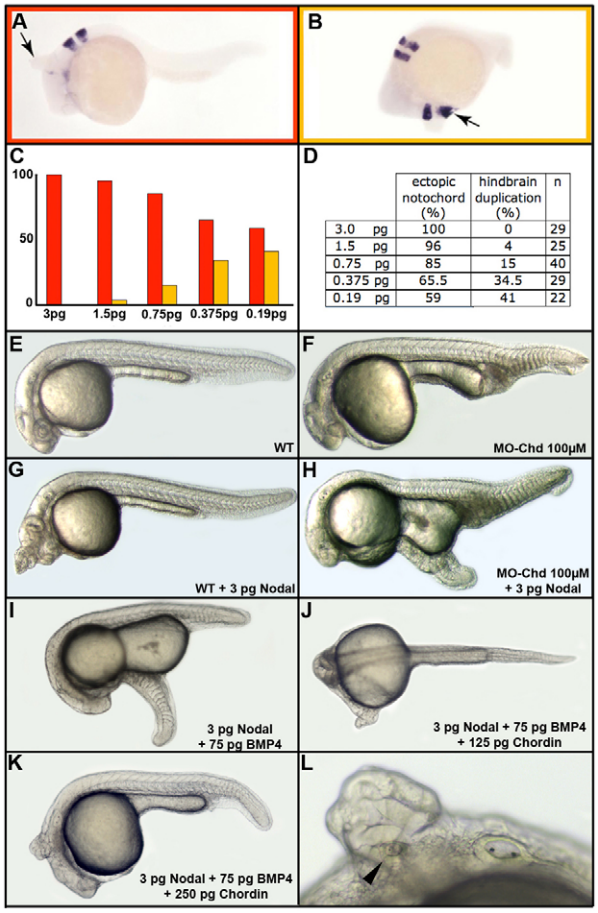

Fig. 6. Interaction between endogenous BMP activity and ectopic Nodal signaling at the animal pole. (**A-L**) Injection of 3 pg *nodal* RNA at the animal pole results in the induction of ectopic axial structures (A, arrow). When lower amounts are injected, partial secondary axes extend posteriorly up to the hindbrain (B, arrow). The bar graph in C, built from data presented in D, presents the percentage of notochord (red) or hindbrain (orange) duplication as a function of the amount of *nodal* RNA injected. Owing to the presence of endogenous BMP activity at the animal pole, the progressive decrease of *nodal* RNA mimics the BMP/Nodal ratio present from the dorsal to the lateral margin. *n*, number of embryos analyzed. Wild type (WT, E) and chordin morphant (MO-Chd, F) embryos. Injection of 3 pg *nodal* RNA at the animal pole of WT embryos results in the formation of an ectopic notochord (G), whereas injection of the same amount in the chordin morphant results in formation of a secondary tail (H). Injection of 3 pg *nodal* and 75 pg *bmp4* RNAs into wild type embryos results in formation of a secondary tail (I). Co-injection of 125 pg *chordin* RNA changes this posterior structure into a secondary trunk (J), whereas co-injection of 250 pg *chordin* RNA results in the formation of posterior head structures (K,L, with a secondary otic vesicle, arrowhead). Lateral view, anterior to the left and dorsal to the top.

it is the ratio of stimulation between BMP and Nodal, and not their absolute value of activity, that controls the A/P identity of the structures organized at the animal pole upon double simulation of these signaling pathways. Our observations support the assumption that injection at the animal pole of different ratios of *nodal* and *bmp* RNAs reproduces, in this presumptive cephalic region, the conditions of relative stimulation for these two signaling pathways at the margin of the blastula and early gastrula (Fig. 4Aa-i).

It is well known that *bmp* RNAs are dynamically expressed at the blastula and gastrula stages and that BMP molecules are present at the animal pole of the blastula embryo and in the ventral part of the animal pole of the gastrula (Hwang et al., 1997; Kishimoto et al., 1997; Dick et al., 2000; Schmid et al., 2000). Therefore, it is possible that, for a low amount of *nodal* RNA injected, the endogenous BMP might interact with this ectopic Nodal activity. We investigated this hypothesis by injecting a range of *nodal* RNA (decreasing from 3 pg to 0.19 pg) and measuring the frequency of embryos that developed a morphologically visible ectopic notochord or secondary hindbrain (revealed by in situ hybridization with *krox20*). Decreasing the amount of *nodal* RNA injected leads to a decrease in the percentage of secondary axial structures and an increase in the percentage of posterior head structures (Fig. 6). It is thus very probable that progressively decreasing the amount of exogenous *nodal* RNA enables us to approach the condition at which the level of exogenous Nodal stimulation becomes close to the endogenous level of BMP activity and thus recreates the conditions we observed when we injected a 1/1 ratio of in-vitro-synthesized *nodal* and *bmp* RNAs. These results are in good agreement with our model of action of BMP and Nodal on the formation of different types of structures along the A/P axis. For a low amount of *nodal*, the BMP/Nodal ratio resulting from the endogenous activity of BMP at the animal pole and from the activity of the injected *nodal* RNA allows posterior head structures to be organized. For a higher amount of *nodal*, the endogenous level of BMP activity is negligible and cells respond to the RNA injection as if Nodal were alone, generating an ectopic notochord and therefore mimicking conditions prevailing at the dorsal blastula margin. These experiments further confirm that the

identity of the structures formed upon stimulation by Nodal and BMP depends on the ratio of their activities. In agreement with this, 3 pg of *nodal* RNA are required for 3 pg of *bmp* RNA to form an ectopic posterior head, whereas 15 times less *nodal* RNA is sufficient to generate the same structures when interacting with the endogenous BMP present at the animal pole. Therefore, it is not the absolute amount of *nodal* RNA but its level of activity relative to that of BMP that determines the category of structures generated.

To substantiate this further, we injected *nodal* RNA into embryos displaying increased endogenous BMP activity at the animal pole as a result of the attenuation of *chordin*, either by morpholino knock down or in a homozygous *dino* (*chordin*) mutant background. Under these conditions, the endogenous BMP activity at the animal pole is increased owing to the lack of Chordin activity emanating from the dorsal marginal region. Moreover, Bmp activity resulting from the induction of *bmp* gene expression following injection of *nodal* at the animal pole is no longer challenged by a concomitant induction of *chordin* expression (observed for a high level of Nodal stimulation at the animal pole). Therefore, injection of *nodal* into this *chordin* morphant/mutant background strongly promotes the activity of the BMP signaling pathway. In these embryos, injection of 3 pg of *nodal* RNA into an animal pole blastomere at the 128-cell stage resulted mainly in the formation of ectopic tails (60% tails, *n*=36; 25% trunk structures, *n*=15; 15% ectopic notochord, *n*=9; Fig. 6H). In a reverse experiment, we decreased BMP activity by co-injecting *chordin* RNA. Under conditions that normally give rise to the formation of secondary tails (Nodal/BMP at a 1/25 ratio; Fig. 6I), co-injection of an increasing amount of *chordin* RNA results in embryos displaying mainly secondary trunk structures (75%, *n*=24, for 125 pg *chordin* RNA; Fig. 6J) or secondary posterior heads (80%, *n*=30, for 250 pg *chordin* RNA; Fig. 6K,L). Therefore, an amount of *nodal* RNA that leads only to the formation of ectopic axial structures when injected in a wild-type embryo induces the formation of caudal structures when injected in an embryo with increased BMP activity at the animal pole. Conversely, attenuation of BMP activity by co-injection of *chordin* with *nodal*/*bmp* RNA in a ratio that gives rise to secondary tails, results in the formation of structures of more anterior identity.

Altogether, this demonstrates that it is not the absolute amount of Nodal, but the relative level of Nodal/BMP activity, that defines the A/P nature of the structures that are organized in response to the double stimulation of embryonic cells by the Nodal and BMP signaling pathways.

DISCUSSION

In this study, we show that each domain of the zebrafish blastula-gastrula margin, regardless of its location along the dorsoventral axis, when transplanted into the animal pole of a host embryo, is able to generate ectopic structures containing cells that originate both from the donor and from the host. Each graft behaves as an organizer [as originally defined by Spemann and Mangold (Spemann and Mangold, 1924)] and is able to recruit cells from the host to organize different parts of the embryonic axis. Altogether, this demonstrates that, not only the most dorsal cells, but also every group of cells belonging to the blastula-gastrula margin has the ability to organize parts of the embryonic axis. Therefore, the blastula-gastrula margin acts as a continuous and global organizer.

In the past, the role of the lateral marginal cells has been analyzed and several reports have described a posteriorizing activity (a change in identity from anterior to posterior character) for the lateral mesendoderm (Woo and Fraser, 1997; Bang et al., 1997; Muhr et al., 1997; Wacker et al., 2004). However, none of these studies has identified the organizing activities – that is, the recruitment of animal pole cells of the host to form a partial secondary axis – of these lateral and dorsolateral marginal territories.

We observe co-linearity between the A/P identity of the structures generated at the animal pole and the dorsoventral position along the margin of the embryo from which the graft was taken. In amphibia, formation of different parts of the embryo along the A/P axis has been suggested to result from the timing of action of the dorsal organizer. The dorsal blastopore lip at the beginning of gastrulation acts as a 'young' organizer and enables the formation of the anterior structures, whereas an 'older' organizer, the dorsal blastopore lip at late gastrula, enables the formation of the posterior structures (Mangold, 1933) (reviewed by Stern, 2005). In zebrafish, we also observe a good correlation between the position of cells along the margin and the time when, owing to convergence movements, they will be located in the embryonic axis. Nevertheless, none of the marginal cells in the lateral and dorsolateral position at the onset of gastrulation contributes to the dorsal margin later in gastrulation. The dorsal margin (the dorsal organizer, which gives rise to the chordamesoderm) is uniquely made from cells deriving from the embryonic shield. As a consequence, lateral and dorsolateral marginal cells will never be part of the dorsal organizer at the late gastrula stage. In addition, the ability to organize the different parts of the body (such as the anterior trunk or tail) is already present in lateral or ventral cells at the blastula stage, and this organizing property appears to be intrinsic to the marginal cells and not related to a particular timing or maturation event. Finally, our observations are not consistent with models predicting the existence of individual and discrete organizers (reviewed by Niehrs, 2004); the organizer function appears continuous along the ventral to dorsolateral margin of the zebrafish embryo.

In the past, particular attention has been given to the dorsal margin, the fish equivalent of the Spemann organizer. However, when this tissue is grafted into a naïve territory (the animal pole), it is unable to organize a secondary axis. Molecular analyses of the early patterning of the blastula-gastrula margin provide a good explanation for this observation. Local inhibition of BMP and Wnt8 activity at the ventral margin mimics the effect of a dorsal marginal graft in a ventral position and results in the formation of a complete secondary axis. Nevertheless, preventing BMP and Wnt activity is not sufficient, per se, to organize the embryonic axis, and the expression of BMP and Wnt inhibitors at the animal pole never induces a secondary axis; therefore, other components or events are required. We know that *bmp2b* and *bmp7* mutants display only head and axial tissues (Kishimoto et al., 1997; Schmid et al., 2000; Dick et al., 2000), whereas *wnt8* loss-of-function results in embryos lacking trunk and tail structures (Lekven et al., 2001). In addition, BMP activity appears to be required in a temporal manner for the growth of posterior tissues (Tucker et al., 2008). It has also been shown that inhibition of fibroblast growth factor (FGF) results in truncation of posterior tissues (Griffin et al., 1995). However, the strongest effect on the formation of structures along the A/P axis is observed when the Activin/Nodal signaling pathway is inhibited (Thisse et al., 2000). Nevertheless, stimulation of animal pole cells by Nodal does not induce a secondary axis but leads to formation of axial tissues, mimicking the effect of grafts containing dorsal marginal cells delivered to the animal pole. In summary, BMP, Wnt, FGF and Nodal are necessary for the formation of posterior structures but are unable, by themselves, to generate ectopic embryonic axes. Creation of embryonic axes comprising cells, tissues and organs from the three germ layers requires simultaneous activation of Nodal and BMP signaling pathways. In a wild-type

embryo, this condition is achieved at the ventral and lateral margin of the blastula and early gastrula, which experiences an almost constant level of Nodal and a gradually increasing level of BMP from dorsolateral to ventral marginal domains. Therefore, reproducing, at the animal pole, the various levels of BMP and Nodal stimulation that normally occur at the blastula-gastrula margin recapitulates the complete organizing activities carried out by this territory and results in generating the different parts of the body, from the posterior head (including the midbrain) to the tip of the tail. We observe that the A/P nature of the structures generated in response to the double stimulation by BMP and Nodal changes gradually, without any obvious discontinuities, and the ectopic structures generated show a gradually more posterior identity as the amount of BMP increases.

In summary, we show that control of the A/P identity of the structures formed in response to BMP and Nodal stimulation is attributable to the relative, and not the absolute, levels of activity of these two signals. Therefore, a given part of the embryonic axis, be it the posterior head, trunk or tail, can be organized upon stimulation of animal pole cells with different amounts of *nodal* and *bmp* RNAs, as long as the *nodal*/*bmp* ratio is kept constant. Any change in the ratio results in a switch in the identity of the structures formed. Maintaining BMP constant and progressively decreasing the level of stimulation of Nodal, or maintaining a constant and high level of Nodal stimulation and increasing the endogenous level of BMP activity (by manipulating the activity of a BMP antagonist), results in a switch from structures normally induced by dorsal marginal cells to structures normally induced by lateral or ventral marginal cells. The best interpretation of these data is that decreasing Nodal activity or increasing BMP activity at the animal pole results in a change in the ratio of Nodal/BMP activity, which appears to be the essential parameter that is coupled to the organizing activities of the different blastula-gastrula marginal domains.

Finally, because they are members of the TGFβ superfamily, it is very possible that BMP and Nodal are similarly affected by environmental, physical, chemical or physiological conditions. As a consequence, owing to the various positive and negative regulatory feedback loops operating between the Nodal and BMP pathways, the maintenance of a correct size for the different parts of the body along the A/P axis is likely to be more easily achieved through the control of the ratio of activity of these two signaling pathways than through independent control of their absolute levels of stimulation.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/22/3811/DC1

References

Agathon, A., Thisse, C. and Thisse, B. (2003). The molecular nature of the zebrafish tail organizer. *Nature* **424**, 448-452.

Bang, A. G., Papalopulu, N., Kintner, C. and Goulding, M. D. (1997). Expression of Pax-3 is initiated in the early neural plate by posteriorizing signals produced by the organizer and by posterior non-axial mesoderm. *Development* **124**, 2075-2085.

Beddington, R. S. and Robertson, E. J. (1998). Anterior patterning in mouse. *Trends Genet.* **14**, 277-284.

Dal-Pra, S., Fürthauer, M., Van-Celst, J., Thisse, C. and Thisse, B. (2006). Noggin 1 and follistatin-like 2 function redundantly to chordin to antagonize BMP activity. *Dev. Biol.* **298**, 514-526.

De Robertis, E. M. (2006). Spemann's organizer and self regulation in amphibian embryos. *Nat. Rev. Mol. Cell. Biol.* **7**, 296-302.

Dick, A., Hild, M., Bauer, H., Imai, Y., Maifeld, H., Schier, A. F., Talbot, W. S., Bouwmeester, T. and Hammerschmidt, M. (2000). Essential role of Bmp7 (snailhouse) and its prodomain in dorsoventral patterning of the zebrafish embryo. *Development* **127**, 343-354.

Erter, C. E., Solnica-Krezel, L. and Wright, C. V. (1998). Zebrafish nodal-related 2 encodes an early mesendodermal inducer signaling from the extraembryonic yolk syncytial layer. *Dev. Biol.* **204**, 361-372.

Feldman, B., Gates, M. A., Egan, E. S., Dougan, S. T., Rennebeck, G., Sirotkin, H. I., Schier, A. F. and Talbot, W. S. (1998). Zebrafish organizer development and germ-layer formation require nodal-related signals. *Nature* **395**, 181-185.

Fürthauer, M., Thisse, B. and Thisse, C. (1999). Three different noggin genes antagonize the activity of Bone Morphogenetic Proteins in the zebrafish embryo. *Dev. Biol.* **214**, 181-196.

Fürthauer, M., Van Celst, J., Thisse, C. and Thisse, B. (2004). FGF signalling controls the dorso-ventral patterning of the zebrafish embryo. *Development* **131**, 2853-2864.

Glinka, A., Wu, W., Onichtchouk, D., Blumenstock, C. and Niehrs, C. (1997). Head induction by simultaneous repression of Bmp and Wnt signalling in Xenopus. *Nature* **389**, 517-519.

Griffin, K., Patient, R. and Holder, N. (1995). Analysis of FGF function in normal and no tail zebrafish embryos reveals separate mechanisms for formation of the trunk and the tail. *Development* **121**, 2983-2994.

Harland, R. and Gerhart, J. (1997). Formation and function of Spemann's organizer. *Annu. Rev. Cell. Dev. Biol.* **13**, 611-667.

Hwang, S.-P. L., Tsou, M.-F., Lin, Y.-C. and Liu, C.-H. (1997). The zebrafish BMP4 gene: sequence analysis and expression pattern during embryonic development. *DNA Cell Biol.* **16**, 1003-1011.

Jones, C. M., Kuehn, M. R., Hogan, B. L., Smith, J. C. and Wright, C. V. (1995). Nodal-related signals induce axial mesoderm and dorsalize mesoderm during gastrulation. *Development* **121**, 3651-3662.

Kimura, C., Yoshinaga, K., Tian, E., Suzuki, M., Aizawa, S. and Matsuo, I. (2000). Visceral endoderm mediates forebrain development by suppressing posteriorizing signals. *Dev. Biol.* **225**, 304-321.

Kinder, S. J., Tsang, T. E, Wakamiya, M., Sasaki, H., Behringer, R. R., Nagy, A. and Tam, P. P. (2001). The organizer of the mouse gastrula is composed of a dynamic population of progenitor cells for the axial mesoderm. *Development* **128**, 3623-3634.

Kishimoto, Y., Lee, K. H., Zon, L., Hammerschmidt, M. and Schulte-Merker, S. (1997). The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. *Development* **124**, 4457-4466.

Knoetgen, H., Viebahn, C. and Kessel, M. (1999). Head induction in the chick by primitive endoderm of mammalian, but not avian origin. *Development* **126**, 815-825.

Lekven, A. C., Thorpe, C. J., Waxman, J. S. and Moon, R. T. (2001). Zebrafish wnt8 encodes two wnt8 proteins on a bicistronic transcript and is required for mesoderm and neurectoderm patterning. *Dev. Cell* **1**, 103-114.

Lustig, K. D., Kroll, K., Sun, E., Ramos, R., Elmendorf, H. and Kirschner, M. W. (1996). A Xenopus nodal-related gene that acts in synergy with noggin to induce complete secondary axis and notochord formation. *Development* **122**, 3275-3282.

Mangold, O. (1933). Über die Induktionsfähighkeit der verschiedenen Bezirke der Neurula von Urodelen. *Naturwissenshaften* **21**,761-766.

Muhr, J., Jessell, T. M. and Edlund, T. (1997). Assignment of early caudal identity to neural plate cells by a signal from caudal paraxial mesoderm. *Neuron* **19**, 487-502.

Niehrs, C. (2001). The Spemann organizer and embryonic head induction. *EMBO J.* **20**, 631-637.

Niehrs, C. (2004). Regionally specific induction by the Spemann-Mangold organizer. *Nat. Rev. Genet.* **5**, 425-434.

Niehrs, C., Kazanskaya, O., Wu, W. and Glinka, A. (2001). Dickkopf1 and the Spemann-Mangold head organizer. *Int. J. Dev. Biol.* **45**, 237-240.

Nikaido, M., Tada, M., Saji, T. and Ueno, N. (1997). Conservation of BMP signaling in zebrafish mesoderm patterning. *Mech. Dev.* **61**, 75-88.

Oppenheimer, J. M. (1936). Transplantation experiments on developing teleosts (*Fundulus* and *Perca*). *J. Exp. Zool.* **72**, 409-437.

Pera, E. M. and Kessel, M. (1997). Patterning of the chick forebrain anlage by the prechordal plate. *Development* **124**, 4153-4162.

Piccolo, S., Agius, E., Leyns, L., Bhattacharyya, S., Grunz, H., Bouwmeester, T. and De Robertis, E. M. (1999). The head inducer Cerberus is a multifunctional antagonist of Nodal, BMP and Wnt signals. *Nature* **397**, 707-710.

Renucci, A., Lemarchandel, V. and Rosa, F. (1996). An activated form of type I serine/threonine kinase receptor TARAM-A reveals a specific signalling

pathway involved in fish head organiser formation. *Development* **122**, 3735-3743.
Sampath, K., Rubinstein, A. L., Cheng, A. M., Liang, J. O., Fekany, K., Solnica-Krezel, L., Korzh, V., Halpern, M. E. and Wright, C. V. (1998). Induction of the zebrafish ventral brain and floorplate requires cyclops/nodal signalling. *Nature* **395**, 185-189.
Saude, L., Wooley, K., Martin, P., Driever, W. and Stemple, D. L. (2000). Axis-inducing activities and cell fates of the zebrafish organizer. *Development* **127**, 3407-3417.
Schmid, B., Fürthauer, M., Connors, S. A., Trout, J., Thisse, B., Thisse, C. and Mullins, M. C. (2000). Equivalent genetic roles for *bmp7/snailhouse* and *bmp2b/swirl* in dorsoventral pattern formation. *Development* **127**, 957-967.
Seiliez, I., Thisse, B. and Thisse, C. (2006). FoxA3 and Goosecoid promote anterior neural fate through inhibition of Wnt8a activity before the onset of gastrulation. *Dev. Biol*. **290**, 152-163.
Shimizu, T., Yamanaka, Y., Nojima, H., Yabe, T., Hibi, M. and Hirano, T. (2002). A novel repressor-type homeobox gene, ved, is involved in dharma/bozozok-mediated dorsal organizer formation in zebrafish. *Mech. Dev.* **118**, 125-138.
Spemann, H. and Mangold, H. (1924). Über Induktion von Embryoalanlagen durch Implantation artfremder Organisatoren. *Wilhem Roux Arch. Ent. Mech. Org.* **100**, 599-638.
Stern, C. D. (2005). Neural induction: old problem, new findings, yet more questions. *Development* **132**, 2007-2021.
Thisse, B., Wright, C. V. and Thisse, C. (2000). Activin- and Nodal-related factors control antero-posterior patterning of the zebrafish embryo. *Nature* **403**, 425-428.
Thisse, B., Pflumio, S., Fürthauer, M., Loppin, B., Heyer, V., Degrave, A., Woehl, R., Lux, A., Steffan, T., Charbonnier, X. Q. and Thisse, C. (2001). Expression of the zebrafish genome during embryogenesis. ZFIN Direct Data Submission (http://zfin.org/cgi-bin/webdriver?MIval=aa-ZDB_home.apg).
Thisse, C. and Thisse, B. (1999). Antivin, a novel and divergent member of the TGFbeta superfamily, negatively regulates mesoderm induction. *Development* **126**, 229-240.
Thisse, C. and Thisse, B. (2008). High resolution in situ hybridization on whole-mount zebrafish embryo. *Nat. Prot.* **3**, 59-69.
Tucker, J. A., Mintzer, K. A. and Mullins, M. C. (2008). The BMP signaling gradient patterns dorsoventral tissues in a temporally progressive manner along the anteroposterior axis. *Dev. Cell* **14**,108-119.
Wacker, S. A., Jansen, H. J., McNulty, C. L., Houtzager, E. and Durston, A. J. (2004). Timed interactions between the Hox expressing non-organiser mesoderm and the Spemann organiser generate positional information during vertebrate gastrulation. *Dev. Biol.* **268**, 207-219.
Woo, K. and Fraser, S. E. (1997). Specification of the zebrafish nervous system by nonaxial signals. *Science* **277**, 254-257.
Yasuo, H. and Lemaire, P. (2001). Role of Goosecoid, Xnot and Wnt antagonists in the maintenance of the notochord genetic programme in Xenopus gastrulae. *Development* **128**, 3783-3793.