Anterior-posterior axis formation in Xenopus laevis

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CHAPTER 3

The role of the Spemann organizer in anterior-posterior patterning of the trunk

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Abstract

The formation of the vertebrate body axis during gastrulation strongly depends on a dorsal signaling centre, the Spemann organizer as it is called in amphibians. This organizer affects embryonic development by self-differentiation, regulation of morphogenesis and secretion of inducing signals. Whereas many molecular signals and mechanisms of the organizer have been clarified, its function in anterior-posterior pattern formation remains unclear. We dissected the organizer functions by generally blocking organizer formation and then restoring a single function. In experiments using a dominant inhibitory BMP receptor construct (tBr) we find evidence that neural activation by antagonism of the BMP pathway is the organizer function that enables the establishment of a detailed anterior-posterior pattern along the trunk. Conversely, the exclusive inhibition of neural activation by expressing a constitutive active BMP receptor (*hAlk-6*) in the ectoderm prohibits the establishment of an anterior-posterior pattern, even though the organizer itself is still intact. Thus, apart from the formerly described separation into a head and a trunk/tail organizer, the organizer does not deliver positional information for anterior-posterior patterning. Rather, by inducing neurectoderm, it makes ectodermal cells competent to receive patterning signals from the non-organizer mesoderm and thereby enable the formation of a complete and stable AP pattern along the trunk.

Introduction

In all vertebrates, the formation of the main body axis begins with the generation of an organizing centre. Interactions between this organizing centre and surrounding tissues during gastrulation generate a basic body plan. Since the initial discovery of this structure in the dorsal blastopore lip of amphibians (the Spemann organizer, hereafter called the organizer (Spemann and Mangold, 1924)), comparable organizing centers have been found in other vertebrate groups (i.e. the node in mouse, Hensen´s node in chicken, embryonic shield in zebrafish (Joubin and Stern, 2001; Niehrs, 2004). Many of their functions have been identified and the molecular pathways involved have been characterized. In the amphibian *Xenopus laevis* the functions of the organizer have been divided into three categories (Harland and Gerhart, 1997). First, self-differentiation of the organizer generates a variety of mesodermal and endodermal tissues, including head mesoderm, notochord, and pharyngeal endoderm. Second, the organizer performs morphogenetic movements and in addition induces them in adjacent cells (e.g. convergence and extension in the presumptive notochord and in the somitic mesoderm). The timing of mesodermal and endodermal internalization also depends on signals from the organizer. Bottle cell formation, involution and vegetal rotation start up to two hours earlier on the organizer side than on the opposite side (Shih and Keller, 1994; Ibrahim and Winklbauer, 2001; Winklbauer and Schürfeld, 1999). Third, the organizer secrets signals which affect all three germ layers of the developing embryo. Most of these signals have been found to antagonize ventralizing signals like BMPs, Wnts, and Nodals (for review (Niehrs, 1999; De Robertis and Kuroda, 2004)).

It has been clear for a long time that the organizer is of crucial importance for the development of the anterior-posterior (AP) axis (Spemann and Mangold, 1924). In an early model, it was postulated that different portions of the organizer mediate different positional values along the AP axis (Mangold, 1933; Eyal-Giladi, 1954). Head, trunk, and tail

organizing areas have been described in the Xenopus organizer (Zoltewicz and Gerhart, 1997; Lane and Keller, 1997) and other vertebrates (Agathon et al., 2003; Kaneda et al., 2002).Vertical signals from different portions of the internalized organizer mesoderm to the overlying prospective neurectoderm have been suggested for regulation of a few, very anterior patterning genes (Hemmati-Brivanlou et al., 1990; Blitz and Cho, 1995). In addition gradients of secreted molecules (FGFs, Wnts, retinoic acid) have been postulated to act in a planar way along the AP axis to define more posterior values (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Kiecker and Niehrs, 2001; Durston et al., 1989).

Pieter Nieuwkoop and his colleagues proposed an alternative model. They postulated that, neurectoderm of an anterior character is induced via an initial "activation" step (i.e. the actual neural induction or neuralization),. In a subsequent "transformation" step this anterior neurectoderm is gradually modified to make more posterior regions of the central nervous system (Nieuwkoop, 1952; Nieuwkoop and van Nigtevecht, 1954).

A recent modification of this model is the "three-step model" (Stern, 2001; Fraser and Stern, 2004). "Activation" establishes an anterior pre-neural state. In a "stabilization" step this territory is then maintained and converted into the definitive forebrain/midbrain. Parts of the neural territory are posteriorized by "transformation". Signals involved in the activation step are organizer derived (Wnt antagonists, BMP antagonists, and Nodal signaling (Niehrs, 2004; Stern, 2005; Vonica and Brivanlou, 2006)), as well as non-organizer derived (FGF and RA (Delaune et al., 2004; Londin et al., 2005; Wilson et al., 2000; Linker and Stern, 2004; Albazerchi and Stern, 2006)). Stabilization signals for the pre-neural state are expected to originate from the organizer (Albazerchi and Stern, 2006). Growing evidence indicates that transforming signals in different species originate from the non-organizer portion of mesoderm (Bang et al., 1997; Bang et al., 1999; Muhr et al., 1997; Muhr et al., 1999; Gaunt et al., 1999; Gould et al., 1998; Kolm et al., 1997; Wacker et al., 2004a; Woo and Fraser, 1997). However, the organizer is at least involved in an initial separation of head and trunk (Glinka et al., 1997; Niehrs, 1999). It remains unclear, whether making a more detailed AP pattern is a direct function of the organizer itself, or whether the organizer merely has a facilitating function in the establishment of the AP axis, different regions of which develop independent of an organizer prepattern (Wacker et al., 2004a; Ober and Schulte-Merker, 1999; Ang and Rossant, 1994).

Several of the organizer functions mentioned above are crucial for AP axis formation. First, morphogenetic movements in both mesoderm and neurectoderm are important for axis formation, even though their involvement in AP patterning needs further analysis (Ninomiya et al., 2004; Elul et al., 1997). Second, the creation of a dorsoventral polarity by secreted signals from the organizer could have a direct effect on non-organizer mesoderm and could create a prepattern in this mesoderm as is possibly indicated by the differential expression of components of the Wnt pathway (Salic et al., 1997). This mesodermal prepattern could then be the basis for neural transformation. Third, the organizer might induce ectodermal cells to be competent for transformation signals. Differential competence could even result in AP patterning as it has been postulated for zebrafish (Koshida et al., 1998). And fourth, the organizer is involved in the stabilization step for neurectoderm (Stern, 2001; Albazerchi and Stern, 2006).

To get a better understanding of the role of the organizer in AP patterning we dissected the organizer functions and manipulated them separately. Since several molecular pathways

involved in different functions of the organizer are well characterized, single organizer functions can be knocked down in certain tissues or whole embryos. Alternatively, the formation of an organizer can be efficiently prevented by UV irradiation (Scharf and Gerhart, 1983). A single organizer function can then be restored without reestablishment of others.
In this paper, we demonstrate that neural activation is the function of the organizer, which is crucial for the establishment of a detailed AP pattern. Prevention of neural activation by localized application of the constitutively active BMP receptor *hAlk-6* disables the expression of AP patterning genes. Restoration of neural activation by localized application of the dominant inhibitory BMP receptor tBr and *FGF-4* under conditions, where all other organizer functions are absent, i.e. ventralized embryos or on the ventral side of normal embryos, enabled the expression of AP marker genes. We conclude that neural activation by the organizer makes ectodermal cells competent to respond to the transforming AP patterning signals originating from the non-organizer mesoderm, thereby enabling the stable formation of posterior AP positional values, which are characteristic for the trunk.

Results

Signals from the organizer are involved in neural activation, but not in transformation

We have recently published the observation that organizer mesoderm alone is not sufficient to induce the expression of posterior positional markers in the neuroectoderm, indicating that neural transformation is disturbed or even absent (Wacker et al., 2004a). Here we use the so-called wrap assay to analyze in detail the effects of the organizer for AP patterning of the ectoderm. For this wrap assay, pieces of mesoderm are recombined with ectoderm (Figure 1 A). After several hours these wraps are fixed, used for in situ hybridization and then bisected across the mesodermal implants (Figure 1 B, C). Due to tissue separation (Wacker et al., 2000), mesodermal and ectodermal cells do not intermingle during the experiment as is shown here with fluorescence labeling (different mesodermal tissues were labeled in red and green using converted and not converted EosFP (Wiedenmann et al., 2004; Wacker et al., 2006) and then implanted into unlabeled ectoderm, Figure 1 C).
For our purpose, pieces of organizer mesoderm were wrapped in ectodermal animal caps. This exclusive recombination of the donor and the recipient of neural activation signals reduces interfering effects from other sources. Wraps were analyzed with in situ hybridization for pan-neural markers and AP patterning genes, at a time when sibling embryos were at neurula stages. A set of pan-neural markers was expressed in the ectoderm of the wraps indicating that neural tissue was induced (*Nrp-1*, *Sox-2*, *Sox-3*, Figure 1 D-F). In addition, the anterior gene *Xotx-2* is expressed (Figure 1 G). *En-2*, which is normally detected in the midbrain-hindbrain border, is expressed rudimentarily (Figure 1 H). More posterior genes that are normally expressed in the hindbrain or in the spinal cord were not found. This was true for the hindbrain marker *Krox-20* (Figure 1 I), and a set of Hox genes, representing different AP positions (*Hoxd-1*, *Hoxc-6*, *Hoxb-9*, Figure 1 J-L).
We conclude that in wraps, which contain exclusively organizer mesodermal implants, neural tissue of anterior quality is induced, whereas positions behind the midbrain are absent, indicating a failure of neural transformation. This is in correspondence with the observation that transforming non-organizer signals are necessary for AP patterning.

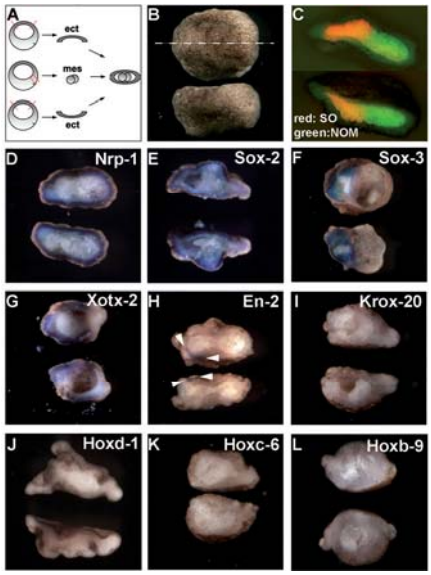

Fig. 1 The Spemann organizer induces a rudimentary AP pattern in Wrap assays. A In the Wrap assay small mesodermal explants (mes, e.g. organizer or non-organizer mesoderm) are wrapped into ectodermal animal caps (ect) to analyze inductive events. B A wrap in topview and lateral view. For the analysis Wraps are bisected after in situ hybridization across the implants (dashed line). C Both halves of a bisected Wrap. Vital staining of two mesodermal implants (red; organizer mesoderm (SO), green; non-organizer mesoderm (NOM)) in unlabeled ectoderm show that the tissues do not intermingle. D, E, F The general neural markers *Nrp-1*, *Sox-2*, and *Sox-3* are activated in Wraps containing exclusively organizer implants. G The forebrain marker X*otx-2* is induced in the ectoderm of wraps by an organizer implant. H *En-2*, a marker for the midbrain-hindbrain boundary, is weakly induced (arrowheads) by the organizer. I *Krox-20*, which is normally expressed in the rhombomeres 3 and 5 of the hindbrain, is not activated by the organizer. J, K, L The Hox genes *Hoxd-1*, *Hoxc-6*, and *Hoxb-9*, which label different positions along the AP axis are not induced in Wraps containing exclusively organizer mesoderm.

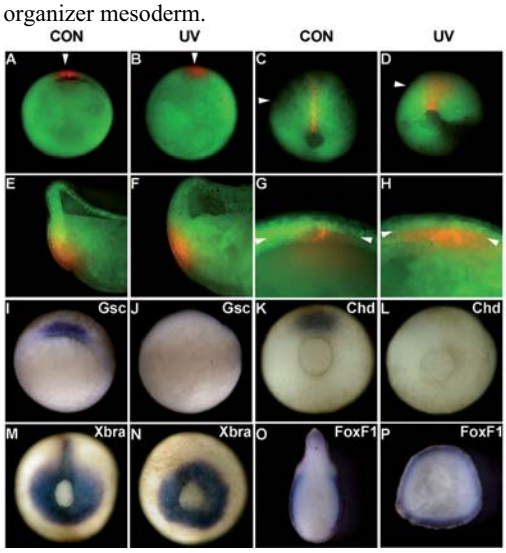

Fig. 2 UV treatment results in embryos without an organizer. Embryos were injected with EosFP. The EosFP in a small region of the marginal zone was converted at the beginning of gastrulation. A An untreated control embryo (CON) with a conversion in the organizer region. B An UV treated embryo (UV) with a corresponding conversion of the marginal zone. C Shape of the conversion at late gastrulation in an untreated control embryo. D Shape of the conversion at late gastrulation in an UV treated embryo. E Bisection of the control embryo shown in A. F Bisection of the UV treated embryo shown in B. G Bisection of the control embryo shown in C. Arrowheads indicate Brachet's cleft between not involuted and involuted tissue. H Bisection of the control embryo shown in D. Arrowheads indicate Brachet's cleft. The arrowheads in A-D indicate the plane of bisection. Comparison of gene expression in control embryos and UV treated embryos. **I, J** The expression of the organizer gene *Goosecoid* (Gsc) at early gastrulation in an untreated control (**I**) and in an UV treated embryo (**J**). **K, L** The expression of the organizer gene *Chordin* (Chd) at mid to late gastrulation in an untreated control (**K**) and in an UV treated embryo (**L**). **M** The mesodermal marker *Brachyury* (Xbra) is expressed around the blastopore and in the forming notochord. **N** In UV treated embryos the *Xbra* expression around the blastopore is found, the notochordal expression is absent. **O** A cross section of an untreated control embryo labelled for lateral plate mesoderm with *FoxF1* (stage 26). **P** A cross section of an UV treated embryo labelelled for the lateral plate mesoderm with *FoxF1* (stage 26).

An efficient knock down of the organizer by UV irradiation

UV irradiation has been found to be an efficient way of ventralizing Xenopus embryos. Prevention of cortical rotation results in a block of the dorsal Wnt pathway and of subsequent organizer formation (Larabell et al., 1997; Scharf and Gerhart, 1983; Vincent and Gerhart, 1987). UV irradiation may therefore be used to obtain embryos lacking an organizer. We characterized these ventralized embryos for aspects of gene expression and aspects of tissue movements to check the absence of the organizer and of its functions. Changes in morphogenesis have been reported in UV ventralized embryos (Mise and Wakahara, 1994). Using lineage labeled embryos, we analyzed, if, except for the organizer specific defects, gastrulation movements still take place. Especially involution, which places mesoderm underneath the ectoderm, might be important for AP patterning. In embryos expressing the convertible fluorescent protein EosFP, a portion of the mesodermal marginal zone was converted from green to red fluorescence at the beginning of gastrulation, as has been described previously (Wacker et al., 2006). These embryos are shown at an early gastrula stage (Figure 2 A, B) and at a late gastrula stage (Figure 2 C, D). The involution of mesoderm takes place in both, in non-treated and UV treated embryos. Cross sections demonstrate that the labeled mesoderm is internalized in both during gastrulation (Figure 2 G, H). Differences were found for the organizer related convergence and extension. Whereas in non-treated embryos the labeled spot elongates extensively (corresponding to convergence and extension of the notochordal precursors, Figure 2 C, G), this was dramatically reduced in UV treated embryos (Figure 2 D, H). The effect on blastopore formation and closure was also organizer related. In non-treated embryos the blastopore first forms on the dorsal side and then consecutively in lateral and ventral regions. In UV treated embryos the blastopore appeared as a complete ring at the time, when it formed on the ventral side in non-treated embryos. The blastopore then closed at about the same time in both, non-treated and UV treated embryos (not shown). We conclude that, except for the organizer specific morphogenetic movements, gastrulation movements in UV treated embryos are normal.

By marker analysis of UV treated embryos, we find that under our conditions and in agreement with former studies (examples of which are (Pannese et al., 1995; Penzel et al., 1997; Stoetzel et al., 1998)) organizer specific genes are not expressed. Neither goosecoid (shown at early gastrula stages in Figure 2 I, J) nor chordin (shown at late gastrula stages in Figure 2 K, L) are expressed in UV treated embryos, indicating the absence of an organizer during gastrulation. Non-organizer mesoderm is still present as it is shown by the early mesodermal marker brachyury (Figure 2 M, N). At tadpole stages this mesoderm was identified mainly as lateral plate mesoderm (expression of FoxF1 in cross sections, figure 2 O, P). Other mesodermal tissues are strongly reduced or absent (notochordal brevican, somitic myoD, intermediate mesodermal XWnt-8, Supplement 1). As expected, the expression of neural and neural crest markers is reduced and correspondingly, the expression of an epidermal marker is expanded (Supplement 1). A set of AP positional markers is not detectable in UV ventralized embryos (Supplement 1 and (Wacker et al., 2004a)). This indicates that beside the organizer-non-organizer asymmetry the AP pattern is also absent in UV treated embryos.

Overall we find that organizer independent prerequisites for AP patterning (i.e. non-organizer mesoderm formation and appropriate morphogenetic movements, see also (Wacker et al., 2004a)) were not affected by the UV treatment, whereas organizer specific

genes and functions were absent. This delivers the optimal experimental preconditions for our following experiments.

Neural activation enables AP patterning by transformation without an organizer

One of the organizer functions is neural activation. To study the importance of this function for AP pattern formation, we prevented formation of the organizer and thereby its functions by UV irradiation of early embryos. Then we neurally activated their ectoderm by injection of a combination of mRNAs encoding for a dominant negative BMP receptor (tBr) and a very low dose of *FGF-4*. This treatment suppressed epidermal fate in the ectoderm and efficiently promoted a neural fate (Linker and Stern, 2004; Delaune et al., 2004). To confirm that the injected mRNAs exclusively led to neural activation, we injected the same amounts of either tBr or *FGF-4* mRNA's alone. This gave the same results as described in (Delaune et al., 2004). Reduction of BMP signalling caused ectoderm to adopt a neural crest fate, very low doses of *FGF-4* mRNA did not cause mesoderm induction or posteriorization (not shown).

Embryos treated in this way were analyzed for mesodermal and ectodermal marker gene expression as well as for markers that show AP patterning. Since completely UV ventralized embryos do not have an organizer-non-organizer pattern, the resulting effects should be radially symmetric and can therefore be distinguished from the effects caused by partial ventralization, which should still show a organizer-non-organizer asymmetry. The absence of organizer both in ventralized embryos and in ventralized and neurally activated embryos, was confirmed by the reduction of staining of the organizer genes *Goosecoid* at early gastrulation (Figure 3 A-C) and of *Chordin* at later gastrulation (Figure 3 D-F). The *Xbra* expression pattern supported this, since the presumptive notochordal *Xbra* expression is absent in both, UV treated embryos and UV treated embryos injected with tBr and *FGF-4* mRNAs (Figure 3 G-I). The non-organizer mesodermal expression domain of *Xbra* is still present and even expanded in ventralized and neurally activated embryos, demonstrating that there was no reduction of non-organizer mesoderm (Figure 3 G-I). The ectoderm was analyzed for the expression of the neural marker *Sox-2*. Neural fate, which is absent in UV treated embryos, is brought back by the injection of tBr and *FGF-4* mRNAs (Figure 3 J-L). However, organizer-non-organizer asymmetry is not restored, since *Sox-2* has a radially symmetric expression. Concurrently the expanded epidermal fate (analyzed with XK81A1) in UV treated embryos is radially suppressed by additional injection of the animal cap with the mRNAs for tBr and *FGF-4* (not shown).

AP patterning, which is absent in UV treated embryos, is restored in UV treated embryos after injection of tBr and *FGF-4* as the expression of Hox genes shows. The expression patterns of four different Hox genes (*Hoxd-1*, *Hoxc-6*, *Hoxa-7*, and *Hoxb-9*) were compared in non-treated embryos, in UV treated embryos, and in UV treated and tBr and FGF-4 injected embryos. The characteristic patterns of these Hox genes along the AP axis are shown in non-treated embryos at early neurula stages (Figure 3 M, P) and late neurula stages (Figure 3 S, V). After UV irradiation, Hox gene expression is drastically reduced or absent (Figure 3 N, Q, T, W). The injection of mRNAs for tBr and *FGF-4* into ectodermal precursors enables UV ventralized embryos to express these Hox genes again in their normal AP order (Figure 3 O, R, U, X). In contrast to non-treated embryos, the expression patterns are not restricted to the dorsal side, but appear in a radially symmetric pattern at their correct positions along the AP axis.

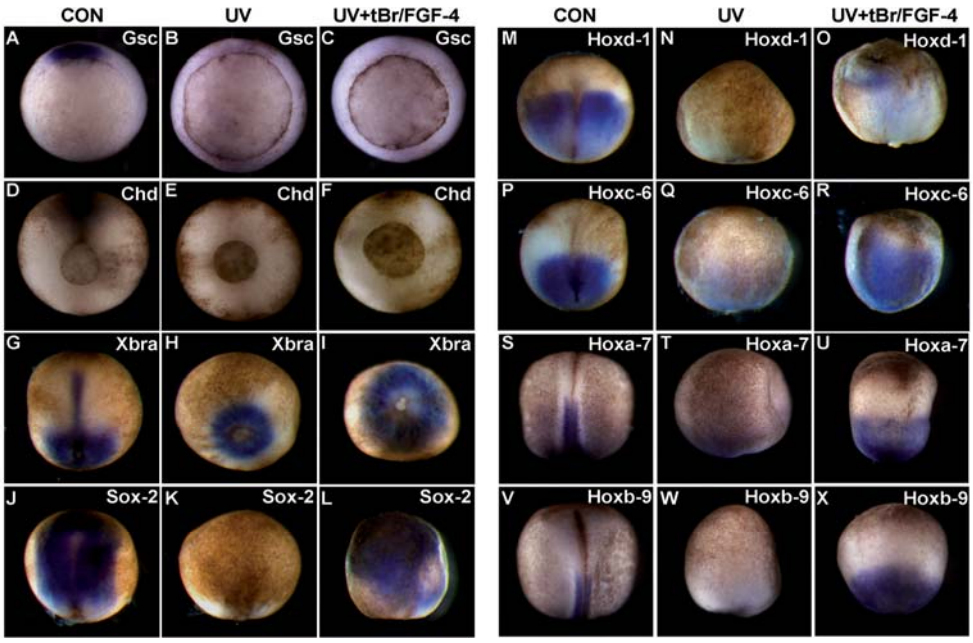

Fig. 3 Restoration of neural activation by injection of tBr and *FGF-4* in predominantly ectodermal precursors of UV treated embryos results in AP patterning of embryos without Spemann organizer. The first column shows expression of different markers in untreated control embryos (CON). The second column shows expression of these markers in UV treated embryos (UV). The third column shows expression of these markers in UV treated embryos, which were animally injected with mRNAs for tBr and *FGF-4* (UV+tBr/FGF-4). A, B, C Vegetal view of early gastrula embryos stained for the organizer gen *Goosecoid* (Gsc). In UV treated embryos gsc is not expressed. Its expression is not restored by the animal injection of tBr and FGF-4, indicating the remaining absence of an organizer. D, E, F Vegetal view of stage 11.5 embryos stained for *Chordin* (Chd), which normally is expressed in the organizer and the overlying neural floor plate. In UV treated embryos it is absent and not restored after tBr and *FGF-4* mRNA injection into the animal pole, indicating the remaining absence of the organizer. G, H, I Expression of the mesodermal marker *Brachyury* (Xbra) at stage 12.5 in the marginal zone around the blastopore and in the prospective notochord (arrowhead). The notochordal expression is absent in UV treated embryos and not restored after injection of tBr and *FGF-4* mRNAs, again indicating the absence of the organizer. The non-organizer expression around the blastopore remains. J, K, L *Sox-2* expression demarcates the neural plate in control embryos, and indicates the absence of neurectoderm in UV treated embryos (stage 15). After injection of tBr and *FGF-4* into the animal region of UV treated embryos, almost all ectoderm shows *Sox-2* expression. M, N, O *Hoxd-1*, which is expressed up to the level of the posterior portion of hindbrain, is absent in UV treated embryos, but is mildly restored after injection of tBr and *FGF-4* mRNAs in ectodermal precursors of UV treated embryos (stage 15). P, Q, R *Hoxc-6*, which is expressed along the spinal cord, is depleted in UV treated embryos. Expression is restored after injection of tBr and *FGF-4* mRNA in the animal blastomeres of UV treated embryos (stage 15). S, T, U *Hoxa-7* expression along the spinal cord of control embryos (stage 18) is depleted in UV treated embryos, but is restored after injection of tBr and *FGF-4* mRNA in animal blastomeres of UV treated embryos. V, W, X *Hoxb-9* expression along the posterior spinal cord of control embryos (stage 18) is depleted in UV treated embryos, but is restored after injection of tBr and *FGF-4* mRNA in animal blastomeres of UV treated embryos.

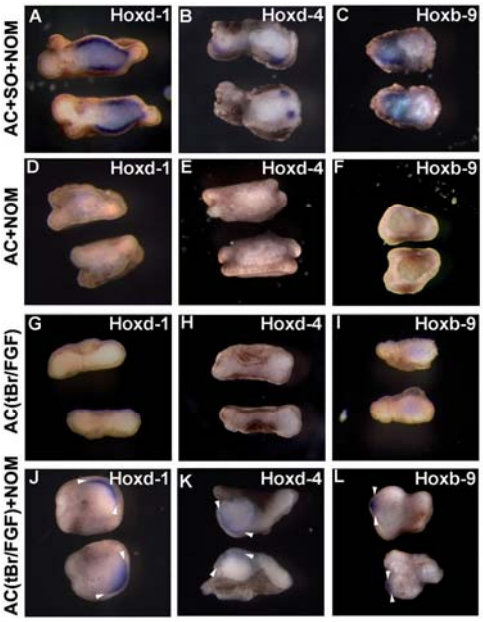

Fig. 4 The Spemann organizer function for AP patterning in Wrap assays can be replaced by neural activation. A, B, C Wraps containing both, organizer and non-organizer mesoderm (AC+SO+NOM) express *Hoxd-1* (A), *Hoxd-4* (B) and *Hoxb-9* (C) in the ectoderm. D, E, F Wraps containing exclusively non-organizer mesoderm (AC+NOM) do not express *Hoxd-1*, *Hoxd-4*, or *Hoxb-9*. The same observation was made for Wraps containing exclusively organizer mesoderm (not shown). G, H, I Neural activation by tBr and *FGF-4* mRNA in the ectoderm of wraps without mesodermal implants (AC(tBr/FGF)) does not result in expression of *Hoxd-1, Hoxd-4,* or *Hoxb-9*. J, K, L tBr and *FGF-4* mRNA injection to neurally activate ectoderm of Wraps containing non-organizer mesoderm (AC(tBr/FGF)+NOM) replaces the organizer function for induction of the expression of *Hoxd-1, Hoxd-4,* or *Hoxb-9* (arrowheads).

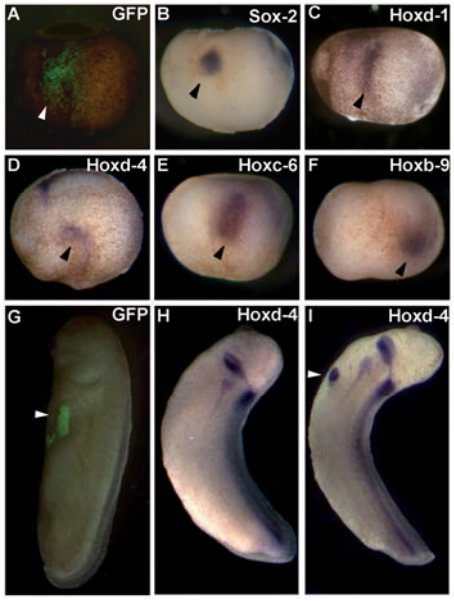

Fig. 5 Ectopic areas of neural activation after injection of tBr and FGF-4 in a ventral animal blastomere at 32-cell stage show AP patterning gene expression independent of the organizer. A, G Lineage tracing with GFP (arrowheads) after coinjection of the mRNAs of tBr, *FGF-4*, and *EGFP*. A shows a ventrolateral view at stage 18. The head is to the left. B The embryo from A after in situ hybridization for the neural marker *Sox-2* shows ectopic ventrolateral *Sox-2* expression (arrowhead). C Ectopic expression of tBr and *FGF-4* results in an ectopic *Hoxd-1* domain at stage 18 (arrowhead). D Ectopic expression of tBr and *FGF-4* results in an ectopic *Hoxd-4* domain at stage 18 (arrowhead). E Ectopic expression of tBr and *FGF-4* results in an ectopic *Hoxc-6* domain at stage 18 (arrowhead). F Ectopic expression of tBr and *FGF-4* results in an ectopic *Hoxb-9* domain at stage 18 (arrowhead). G shows a lateral view at stage 28. The head is up and ventral to the left. The arrowhead indicates the GFP domain. H Lateral view of a normal embryo at stage 30 showing *Hoxd-4* expression. I Lateral view of an injected embryo at stage 30 showing an ectopic ventral patch of *Hoxd-4* expression (arrowhead).

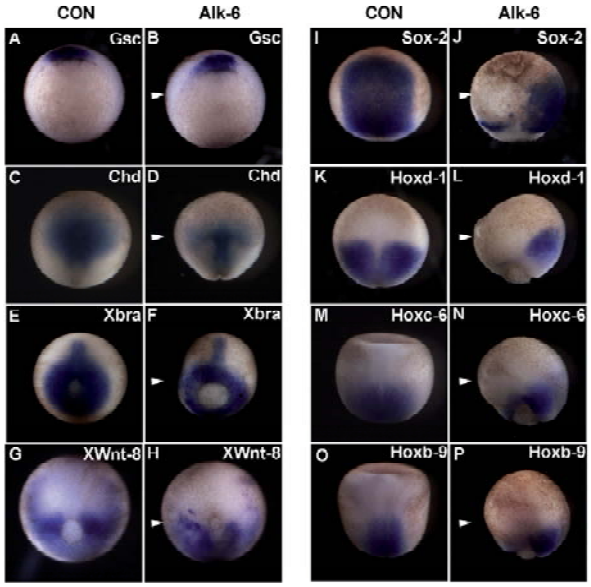

Fig. 6 Inhibition of neural activation in presence of an organizer strongly affects AP pattern formation. mRNA of the constitutively active BMP receptor hAlk-6 was injected into predominantly ectodermal precursors of the left side (arrowheads). A, B. Presence of the early Gsc expression in a non-injected control embryo (CON) and in a hAlk-6 injected embryo (Alk-6). C, D Presence of Chd expression in a non-injected control embryo and in a hAlk-6 injected embryo at the end of gastrulation. E, F Expression of the mesodermal marker Xbra in the organizer tissue of the presumptive notochord, both in a non-injected control, and in a hAlk-6 injected embryo. G, H The posterior marker Xwnt-8 remains in its domain on the injected side. (E,F) I, J The neural marker Sox-2 is expressed in the whole neural plate of control embryos, but it is drastically reduced after hAlk-6 injection. G, H Expression of Hoxd-1. I, J Expression of Hoxc-6. K, L Expression of Hoxb-9.

These results are supported by experiments using wrap assays. A wrap containing both, organizer mesoderm and non-organizer mesoderm expresses Hox genes in the surrounding ectoderm (shown for *Hoxd-1*, *Hoxd-4*, and *Hoxb-9* in Figure 4 A-C). Absence of the organizer in such wraps disables the expression of these Hox genes (Figure D-F). This can be rescued by the injection of tBr and *FGF-4* to get neural activation without an organizer (Figure 4 J-L), even though the injection of these neuralizing factors does not result in Hox gene expression in the absence of mesoderm (Figure 4 G-I).

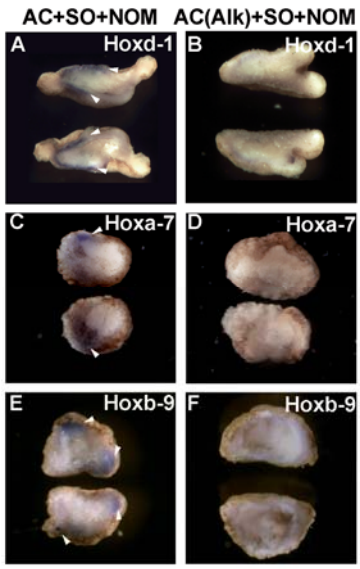

Fig. 7 Inhibition of neural activation by ectodermal expression of the constitutive active BMP receptor *hAlk-6* in wrap assays affects the ectodermal AP patterning. A Expression of *Hoxd-1* (arrowheads) in wraps containing both, organizer and non-organizer mesoderm surrounded by non-injected ectoderm (AC+SO+NOM). B Inhibition of neural activation by injection of *hAlk-6* in the ectoderm of such wraps (AC(Alk)+SO+NOM) results in the repression of *Hoxd-1* expression. C Expression of *Hoxc-6* (arrowheads) in wraps containing organizer and non-organizer mesoderm surrounded by non-injected ectoderm. D Inhibition of neural activation in the ectoderm of such wraps results in repression of *Hoxc-6* expression. E Expression of *Hoxb-9* (arrowheads) in wraps containing organizer and non-organizer mesoderm surrounded by non-injected ectoderm. F Inhibition of neural activation in the ectoderm of such wraps results in the repression of *Hoxb-9* expression.

To further test the importance of neural activation to enable the expression of AP positional markers, we ectopically expressed tBr and *FGF-4* in embryos as far away from the organizer as possible. For this purpose, we injected these mRNAs at the 32-cell stage into the A4-blastomere (i.e. animal tier, opposite to the prospective organizer). Using GFP for lineage tracing, we selected embryos at late neurula stages, which showed the label exclusively outside of the central nervous system (Figure 5 A). Ectopic *Sox-2* expression indicates that ectopic neural activation has taken place (Figure 5 B). In such embryos domains of ectopic Hox gene expression were detected as it is shown for *Hoxd-1* (Figure 5 C), *Hoxd-4* (Figure 5 D), *Hoxc-6* (Figure 5 E), and *Hoxb-9* (Figure 5 F). In tadpoles selected for a ventral localization of the GFP label (Figure 5 G), the coinjection of tBr and *FGF-4* resulted in small ectopic expression domains (shown for *Hoxd-4*, Figure 5 I) compared to embryos GFP-injected without these mRNAs (Figure 5 H).
Overall, we find that neural activation independent of an organizer in UV treated embryos, in wraps, and on the ventral side of normal embryos caused expression of AP positional markers. This demonstrates that AP patterning occurs independently of other organizer functions, when neural activation takes place.

Inhibition of neural activation in presence of all other organizer functions disables neural transformation

If neural activation is a crucial organizer function for AP patterning, then preventing neural activation without affecting other functions of the organizer should result in an inhibition of neural transformation. To test this, formation of neural tissue was inhibited by activation of the BMP signaling pathway in the dorsal ectoderm. This was achieved by expression of a constitutively active BMP receptor (*hAlk-6*) in ectodermal precursors. The *hAlk-6* mRNA was injected into the predominantly ectodermal precursors on one side of each embryo. The non-injected side was used as an internal control. In addition, the expression patterns of non-injected embryos were analyzed to exclude effects of the injected side on the uninjected side. From the lineage fate of the injected cells it can not be excluded that mesodermal cells are affected as well as the ectoderm. Therefore in situ hybridizations detecting organizer gene expression at early and late gastrula stages were performed to exclude an absence of the organizer and accordingly of organizer functions other than neural activation. The expression patterns of *Goosecoid* and *Chordin* demonstrate that the injection of *hAlk-6* mRNA does not abolish organizer formation (Figure 6 A-D). This is supported by the expression of *Xbra* in the prospective notochord, originating from the organizer (Figure 6 E, F). Both the posterior expression domains of *Xbra* (Figure 6 E, F) and of *XWnt-8* (Figure 6 G, H) are only slightly affected, contradicting extensive effects of our treatment on non-organizer mesoderm. However, neural activation is inhibited on the injected side as it is shown by the reduction of the expression of the pan-neural marker *Sox-2* to a rudimentary posterior domain (Figure 6 I, J). Blocking neural activation results in down-regulation of the analyzed Hox genes *Hoxd-1* (Figure 6 K, L), *Hoxc-6* (Figure 6 M, N), and *Hoxb-9* (Figure 6 O, P). This indicates that AP patterning is prevented, if neural activation is not taking place.As an alternative experimental approach we used the wrap assay to show that blocking neural activation in the ectoderm blocked expression of AP patterning genes, even though both organizer and non-organizer mesoderm are present. This approach also completely restricts the effect of *Alk6* injection to the ectoderm and excludes an direct effect of the injection on the mesoderm. We injected *hAlk-6* mRNA into

whole embryos to disable neural activation. Ectodermal animal caps from these embryos were used to make wraps containing both organizer and non-organizer mesoderm from not injected embryos. Wrap assays containing both types of mesoderm normally express different Hox genes in the ectoderm (Figure 7 A, C, E). When *hAlk-6* expressing ectoderm was used to wrap the organizer and non-organizer mesoderm, the expression of Hox genes was completely disabled (Figure 7 B, D, F).
We conclude that prevention of neural activation in embryos and in wraps by expression of *hAlk-6* in the ectoderm disables AP patterning, even though the other organizer functions are still present.

Discussion

The organizer induces anterior parts of the AP pattern

Organizer functions including self-differentiation, morphogenesis, and inductive signaling have been found to be involved in different embryonic events including the formation of an AP pattern (Harland and Gerhart, 1997). The role of the organizer in the formation of an AP pattern is not fully understood. Evidently, embryos without an organizer (e.g., UV ventralized embryos) fail to form an AP pattern, see introduction and Supplement 1). Within the organizer itself, only a very limited AP pattern has been identified, namely a separation in head, trunk, and tail (Zoltewicz and Gerhart, 1997; Glinka et al., 1997; Lemaire and Kodjabachian, 1996). This makes imprinting of a complex AP pattern with many positional values from the organizer to overlying ectoderm unlikely. Two "imprinted" positional values (e.g. head and trunk) could still be a starting position for the subsequent formation of a more distinct pattern in the neurectoderm (Gamse and Sive, 2001). However, this pattern remains incomplete, if influences from non-organizer mesoderm are eliminated (Wacker et al., 2004a; Wessely et al., 2001). In our experiments, the organizer only induces the most anterior positions including forebrain- and midbrain levels. More posterior positions represented by AP marker genes, including *Krox-20* and a set of Hox genes, were not found. Proceeding from the three-step model of AP patterning this demonstrates that the organizer itself mediates activation and stabilization. It does not establish transformation and AP patterning of the trunk.

The organizer function "neural activation" generates ectodermal competence to form an AP pattern along the trunk

Even though the organizer does not directly produce the signals responsible for neural transformation, our experiments demonstrate that at least one of its functions is necessary to enable the ectodermal expression of posterior positional markers (see above and (Wacker et al., 2004a)). The absence of the organizer and its functions disables transformation in whole embryos (e.g. UV ventralized embryos) and in Wrap assays. To analyze which of the organizer functions is responsible for this, we manipulated the function of neural activation (also called neural induction or neuralization (Vonica and Brivanlou, 2006)) without affecting the other functions of the organizer. Neural activation was achieved by manipulating the BMP and FGF signal transduction pathways mainly in the ectoderm as has been described in the recent literature (Linker and Stern, 2004; Delaune et al., 2004). Enabling neural activation in the absence of the organizer leads to the formation of stable neural tissue, which can be transformed by signals not originating from the organizer. We have shown that disabling neural activation without inhibition of other organizer functions is sufficient to prevent the expression of posterior marker genes. Gain of function and loss

of function experiments demonstrate that neural activation and stabilization by the organizer are necessary and sufficient to enable AP patterning of the trunk by organizer independend signals. The observation that under certain conditions AP patterning can occur in the absence of an organizer has been made in the mouse and zebrafish as well, although it was not clear, if the organizer was completely absent in these embryos (Ang and Rossant, 1994; Ober and Schulte-Merker, 1999).

The non-organizer mesoderm and its role in AP pattern formation

Two sources of AP patterning signals resulting in expression of AP positional markers can be distinguished. First, the head portion of the organizer induces markers that are relevant for forebrain and midbrain, e.g. *Xotx-2* or *En-2* (above and (Blitz and Cho, 1995; Hemmati-Brivanlou et al., 1990)). Second, the non-organizer mesoderm has been described in different vertebrates as a signal source, establishing more posterior positional values of the trunk (i.e. hindbrain and spinal cord, for references see introduction). For this second portion of the AP pattern, neural activation is an indispensable element. Ectopic neural activation independent of an organizer allows ectopic expression of AP patterning genes, if non-organizer mesoderm is present. The loss of neural activation prevents expression of AP patterning genes even in presence of both, the organizer and the non-organizer mesoderm.

We recently proposed a model that describes amphibian trunk AP patterning during gastrulation (time space translator model, (Wacker et al., 2004a)). This model describes that AP identities arise in the non-organizer mesoderm in a domain defined by the presence of *Xbra* and BMP signaling (Wacker et al., 2004b). Due to morphogenetic movements, mesodermal cells with particular AP identities leave this domain at different times and move nearer to the organizer. Under influence of the organizer, their AP identities also appear in an adjacent neurectodermal domain. Stripes with different AP identities are thereby created within the neurectoderm along the AP axis. A connection between appearance of an AP pattern and morphogenetic movements has recently been described for chicken (Iimura and Pourquie, 2006), although there it is postulated that the appearance of the Hox genes control morphogenetic movements and not vice versa.

In our model, the organizer has different functions. First it controls morphogenetic movements that are necessary to bring mesodermal cells close to the organizer (i.e. by convergence and extension) and in contact with the ectoderm (by involution). These adjacencies are necessary to allow the expression of AP patterning genes in the ectoderm. From the data presented here, we conclude that a second function of the organizer in our model is neural activation and stabilization, thereby enabling the ectoderm to process transforming signals from the non-organizer mesoderm and generate an AP pattern along the trunk.

Concluding remarks

The central question in this paper is the role of the Spemann organizer in AP patterning of the trunk. The organizer itself does not establish a complete AP pattern, but only a limited stretch from anterior to the midbrain-hindbrain boundary. Our experiments have shown that one essential function of the organizer for AP patterning of the trunk is to make the ectoderm competent to respond to AP patterning signals from other sources. Our approach of bringing back single organizer functions into embryos without an organizer demonstrated that neural activation of the ectoderm is necessary and sufficient to provide this competence of the ectoderm to receive the signals that lead to transformation and formation of a complete and stable AP pattern in the trunk region of the body axis.

Materials and methods

Handling and treating embryos

Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). In vitro fertilization, embryo culture, operation techniques, mRNA injection, and culture of recombined explants were carried out as previously described (Wacker et al., 2000; Winklbauer, 1990). Ventralization with UV light was described previously (Scharf and Gerhart, 1983).

For lineage labelling the light convertible fluorescent protein EosFP was injected into whole embryos. Procedures for injection, conversion and analysis of this protein have been recently described (Wacker et al., 2006). At very early gastrulation the organizer or a corresponding area of the mesodermal marginal zone were converted from green to red. At the end of gastrulation whole embryos or bisected embryos were analyzed.

To prevent neural activation in ectodermal cells, 10 nl of *hAlk-6* mRNA (25 pg/nl) was injected in the two animal blastomeres on the left side of stage 4 embryos. To achieve neural activation, 10 nl of a mix of tBr (25 pg/nl) and *FGF-4* (0.004 pg/nl) was injected into the 4 animal blastomeres of UV treated stage 4 embryos. Alternatively 2 nl of this tBr/*FGF-4* mRNA mix was injected into the A4 blastomere (Dale and Slack, 1987) of a 32 cell normal embryo. To trace injections, GFP mRNA was coinjected. The mRNAs for injection were generated from plasmids: tBr64T, (dominant negative BMP receptor) (Graff et al., 1994); pCS2FGF-4 (made as described in (Delaune et al., 2004)); pCS2ALK6HA (constitutively active *hALK6*) (kind gift from Peter ten Dijke, described in (Wacker et al., 2004b)); pCS2EGFP; and pCS2+MT-d2EosFP (Wacker et al., 2006).

Wrap assays

Microsurgery was carried out using hair knives. Explants and transplantations were done in MBS. The wrap assay is based on previously described experiments (Zoltewicz and Gerhart, 1997; Wacker et al., 2004a). Organizer mesoderm and non-organizer mesoderm was explanted at stage 10. The size of an explant corresponds to an angle of less than 30° of the marginal zone and a height of about 10 epithelial cells. After removing the epithelial layer and keeping these explants for a few minutes in MBS, they were placed between two animal cap explants, which had been cut immediately before to prevent curling. Wraps were cultivated in MBS for about 30 min and then transferred to 10% MBS. They were fixed for in situ hybridization at late gastrula and neurula stages of sibling embryos, when ectodermal expression of the marker genes is known to be strong. For photographing wraps were bisected across the implants.

For fluorescent labelled wraps embryos were injected with EosFP as described before (see above and (Wacker et al., 2006)). At the beginning of gastrulation the EosFP in the organizer domain was converted to red. A piece of converted organizer mesoderm and a piece of labelled, but not converted, non-organizer mesoderm (green) were implanted in unlabelled ectoderm at early gastrulation and analyzed at early neurulation.

Detection of gene expression

Whole mount in situ hybridization (WISH) was performed as previously described (Wacker et al., 2004a). For wrap assays, embryos were cut with a razorblade after WISH. Antisense, DIG-labeled transcripts were prepared from the following plasmids: a 1109 bp *Sox-2* fragment in pBluescript SK(+); cDNA clone AGENCOURT_10482135 (*Sox-3*); pNPG152 (*nrp-1*) (Richter et al., 1990); pBS"HD-anti" (*Xotx-2*) (Blitz and Cho, 1995); a 1500-bp

Engrailed-2 cDNA (*En-2*) (Hemmati-Brivanlou et al., 1991); a 1400-bp *Krox-20* fragment (Bradley et al., 1993); xHoxlab1 (*Hoxd-1*) (Sive and Cheng, 1991); cDNA clone XL094l20 (NIBB) (*Hoxd-4)*;a 998-bp *Hoxc-6* fragment in pGEM1; Xhox-36.1 (*Hoxa-7*) (Condie and Harland, 1987); a 470-bp *Hoxb-9* fragment in pGEM3; pSP73Xbra (Smith et al., 1991); pCS2Chd (Sasai et al., 1994); pCS2Gsc (*Gsc*); pCSWnt8b (*Wnt8*) (Cui et al., 1995); pCS2FoxF1 (Köster et al., 1999); Brevican (*Xbcan*) (Sander et al., 2001); MyoD (Hopwood et al., 1989); a 807 bp fragment containing the ORF of *Xenopus Snail* cloned in pGEMTeasy; a 536 bp fragment from *XK81A1* in pGEM3 (Epidermal Keratin) (Jonas et al., 1989).

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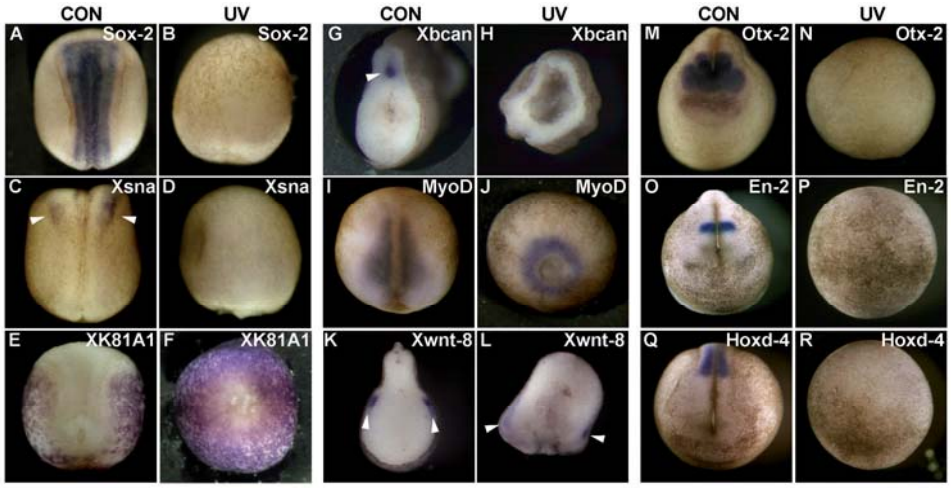

Supplemental figure Marker analysis of UV treated embryos. A The marker *Sox-2* demarkates the neural plate in untreated control embryos (CON) at stage 17 B *Sox-2* is absent in UV treated embryos (UV). C The marker *Xsna* demarcates the neural crest of non-treated control embryos (arrowheads). D In UV treated embryos *Xsna* is not expressed indicating the absence of neural crest. E The epidermal marker *XK81A1* is expressed in non-neural ectoderm of non-treated control embryos. F In UV treated embryos *XK81A1* is expressed in all ectodermal cells indicating the absence of the neural plate. G Cross sections show that brevican (*Xbcan*) is expressed in the notochord (arrowhead) of non-treated control embryos at stage 26, which originates from the organizer. H In cross sections of UV treated embryos of an identical stage no *Xbcan* is found. I The somitic mesoderm marker *MyoD* is expressed in the forming somites on both sides of the dorsal midline in non-treated control embryos at stage 12.5. J In UV treated embryos the *MyoD* expression is reduced to a small domain around the blastopore. K Cross sections of non-treated control embryos at stage 26 show Xwnt-8 expression (arrowheads) in the intermediate mesoderm. L In cross sections of UV treated embryos *Xwnt-8* (arrowheads) is expressed as a stripe around the blastopore indicating that there is still intermediate mesoderm. M, N *Xotx-2* expression in the head mesoderm and the anterior portion of the neural plate is downregulated in UV treated embryos indicating the absence of organizer derived head mesoderm and of neural activation. O In non-treated control embryos (stage 20, anterior view) *En-2* expression demarcates the mid-/hindbrain boundary. P In UV treated embryos the *En-2* expression is absent. Q Hoxd-4 is expressed along the spinal cord in non-treated control embryos (stage 20, anterior view). R In UV treated embryos Hoxd-4 expression was not detected.