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Citation

Bardine, N. (2008, December 3). *Hox in frogs: xenopus reveals novel functions for vertebrate Hox genes*. Retrieved from <https://hdl.handle.net/1887/13306>

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Chapter 6

Vertical signaling involves *Hox* gene expression in the mesoderm

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Abstract

Hox genes are involved in the regionalization of the anterior-posterior axis of all three germ layers. In *Xenopus laevis*, the initial expression of *Hox* genes occurs in a temporally collinear sequence in the gastrula non-organiser mesoderm. Shortly after, the same sequence of *Hox* genes is expressed as a spatially collinear sequence in the overlying and physically connected neurectoderm. The close coordination between these two phases of expression led us to investigate a possible link between the early mesodermal and neurectodermal expression of *Hox* genes. Our results indicate that *Hox* gene expression in the mesoderm is necessary and sufficient to induce expression of the same *Hox* genes in the neural tissue through vertical signaling. The possibility of unconventional transfer of *Hox* proteins between the two layers is being investigated as a mechanism for relaying positional information from the mesoderm to the neurectoderm.

Introduction

Determination of regional specificity along the anteroposterior (A-P) axis of the vertebrate *Xenopus laevis* takes place during gastrulation. During neural induction, neural tissue is, depending on its position along the A-P axis, transformed into one of the three basal central nervous system components, namely forebrain, hindbrain or spinal cord. Taken individually, each of these structures also internally exhibits a high degree of A-P patterning.

This patterning is believed to result from an interaction between the Spemann organizer and the surrounding tissues during gastrulation and is one of the key events leading to the formation of the basic body plan (Spemann and Mangold, 1924; Jansen *et al.*, 2007). The dorsal blastopore lip of the amphibian gastrula was originally shown to trigger the formation of a properly patterned secondary axis when it is grafted into a host embryo and was called the “organizer” (Spemann and Mangold, 1924). Functionally similar structures have been reported in other vertebrates: the zebrafish shield, the chicken Hensen’s node and the mouse node (Stern *et al.*, 2006; Niehrs, 2004; Joubin and Stern, 2001). It has been suggested that different portions of the organizer are responsible for the formation of different structures along the A-P axis and head, trunk and tail organizers have been proposed in different vertebrates (Agathon *et al.*, 2003; Kaneda *et al.*, 2002; Lane and Keller, 1997; Zoltewicz and Gerhart, 1997). Earlier, Mangold had already shown region-specific induction of neural structures in experiments in which grafts of the (mesodermal) archenteron roof from different A-P positions induced tissues that were never anterior to the level of the graft in the donor embryo (Gilbert, 2006; Mangold, 1933). Nieuwkoop proposed an alternative to the “head/trunk/tail organizer” model; the two-step model. In this model, a first “activation” step leads to the formation of anterior neural tissue. Subsequently, this anterior neural tissue is gradually induced to adopt a more posterior character by a “transformation” step (Nieuwkoop, 1952; Nieuwkoop and van Nigtevecht, 1954). It was proposed that two morphogens may be at the basis of these two steps: a dorsal neuralizing activity and a caudal of posteriorizing activity (Nieuwkoop, 1952). Subsequent research has generated much support for the Nieuwkoop model. The proposed neuralizing activity proved to originate from the Spemann organizer and involves secreted molecules such as noggin, chordin or follistatin (which function primarily as BMP antagonists, reviewed in De Robertis and Kuroda, 2004). In contrast to the character of activating signals (namely those from the organizer), there is a classical debate regarding the origin

of the transforming signals. One model proposes that this signal acts within the plane of the neural plate, parallel to the A-P axis of the embryo (so called planar signaling). Another model suggests that the posteriorizing signal originates from the mesodermal tissue underlying the neurectoderm and acts in a plane perpendicular to the embryos (so called vertical signaling) (reviewed in Nieuwkoop, 1997 and Stern *et al.*, 2006).

Candidate signal transduction factors for the posteriorizing step have been proposed to be Fibroblast Growth Factor (FGF) (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Ponwall *et al.*, 1996), retinoic acid (RA) (Kolm *et al.*, 1997; Sive *et al.*, 1990; Durston *et al.*, 1989) and Wnt (Godsave *et al.*, 1998; McGrew *et al.*, 1995). Elevated concentrations of FGF, Wnt or RA at the posterior end of the embryo trigger the formation of relatively posterior positional values in the neurectoderm and each of these factors has been suggested to form a posterior to anterior gradient within the embryo (Doniach *et al.*, 1993; Lamb and Harland, 1995; Kiecker and Niehrs, 2001; Durston *et al.*, 1989; Chen *et al.*, 2001; Gilbert, 2006). There is experimental evidence that planar signaling via gradients does not account for all of the transformation step and is not sufficient to generate the complete anterior posterior pattern observed in the CNS (Grunz *et al.*, 1998; Chen *et al.*, 2000). The use of exogastrula and pseudoexogastrula highlighted the fact that neural markers are only expressed at the border between ectoderm and mesoendoderm, excluding the existence of a very extensive planar signaling. Thus, it is likely that as another source of signals, vertical signaling, also plays an important role in the generation of the A-P pattern of the neural plate.

During gastrulation, mesodermal tissue moves underneath the prospective neurectoderm and has been suggested to pattern the neurectoderm via “vertical signaling” (Gilbert, 2006). It has been suggested that “vertical signaling” is involved in the restriction of *Hox* expression along the A-P axis in the vertebrate *Xenopus* (Poznanski and Keller, 1997), and in the chick embryo (Grapin-Botton *et al.*, 1997; Itasaki *et al.*, 1996). Yet, the exact nature of molecules involved in this process is unclear.

Hox genes are key players in specifying positional information along the A-P axis of the vertebrate embryo. These genes encode a family of transcription factors related to the HOM-C transcription factors in *Drosophila* (reviewed in Lemons and McGinnis, 2006). In the vertebrate *Xenopus laevis*, it has been shown that initial *Hox* expression occurs in a temporally colinear sequence in the marginal zone non-organiser mesoderm (Wacker *et al.*, 2004a).

During gastrulation, cell movements including convergence extension and involution bring these *Hox* expressing cells into the neighbourhood of the Spemann organizer, which itself does not express any *Hox* genes. The proximity to the organizer induces a “stabilisation” of the *Hox* expression pattern within the involuted cells by abrogating the temporally colinear progression and preventing the expression of more posterior genes. Involuting cells thus maintain the *Hox* expression combination they have at the time of interaction with the Spemann organizer signals, while other mesodermal cells will go on to express more posterior *Hox* genes. As a result of this process, the temporal *Hox* gene expression sequence in the marginal zone mesoderm is gradually converted into a spatial *Hox* expression sequence along the developing A-P axis (which is generated in the gastrula by the involuted mesodermal cells) (Wacker *et al.*, 2004a). In addition, the expression of each *Hox* gene that appears in the mesoderm appears shortly afterward in the associated neurectoderm and it seems that there is a direct transfer of information between the two germ layers (Wacker *et al.*, 2004b).

In addition to being transcription factors, *Hox* proteins may mediate intercellular signaling. *Antennapedia*, a *Drosophila* *Hox* protein has been shown to be able to enter neurons as well as other cell types, and it has been demonstrated that its internalization takes place under conditions incompatible with endocytosis (Joliot *et al.*, 1991). This study led to the identification of a 16 amino acid sequence, known as penetratin, capable of and responsible for translocating *Hox* proteins across biological membranes and able to carry diverse cargoes into the cell (reviewed in Joliot and Prochiantz, 2004; Prochiantz, 2003; Letoha *et al.*, 2003; Derossi *et al.*, 1994). The existence of this mechanism in combination with our observations regarding the close synchronisation between neural and mesodermal *Hox* expression, led to the tempting idea that an unconventional transfer of *Hox* proteins from mesoderm to the neurectoderm during gastrulation could constitute the vertical signal responsible for generating the A-P pattern in the spinal cord and hindbrain.

In this study, we investigated the importance of *Hox* genes as part of a vertical signal in a tissue combinatorial assay (wrap assay as in Jansen *et al.*, 2007) that provides a controlled setting for investigating signals from mesoderm to neurectoderm. We found that *Hox* expressing mesoderm is able to provide positional information for the physically connected neurectoderm. Moreover, in these wrap assays, expression in mesodermal tissue of the *Hox* genes that we investigated appears to be necessary for the expression of the same genes in the neurectoderm.

Using recombinant HOX proteins, we found some evidence about the existence of homeodomain proteins uptake by *Xenopus* embryonic cells. Recombinant Hox proteins were synthesised and injected into the extracellular space of the blastocoel. These injections resulted in the same phenotype as obtained by intercellular mRNA injection for the same *Hox* gene in the blastomeres. We demonstrate that *Hox* genes themselves are an essential component of the posteriorizing vertical signal. Our preliminary data point towards a functional role for *Hox* transfer in this process.

Results

***Hox* expression in the mesoderm is necessary and sufficient to induce neural *Hox* expression through vertical signalling**

According to Nieuwkoop's model, neural tissue is induced by activating signals from the organizer. In a second step, this activated neurectoderm is transformed into neural tissues of posterior character (Nieuwkoop, 1952). Recent evidence points towards a role for the non-organizer mesoderm (NOM) in neural patterning. In *Xenopus* it has been suggested that the NOM has a transforming capacity (Kolm and Sive, 1997; Fujii *et al.*, 2002; Wacker *et al.*, 2004b). Similar data have been obtained in the mouse (Gould *et al.*, 1998), the chick (Muhr *et al.*, 1997) and the zebrafish (Woo and Fraser, 1997). We proposed to investigate whether *Hox* expression in the NOM of the gastrula is correlated to *Hox* expression in the neurectoderm and whether it contributes directly to the former's posteriorizing capacity. We used a tissue combinatorial assay, the wrap assay, in which pieces of mesodermal tissues from the Spemann organizer (SO) and from the NOM are wrapped between two animal caps (Fig. 1 A). The main advantage of this wrap assay is that it mimics the embryonic situation with respect to proximity and physical connectivity of Spemann organizer, non organizer mesoderm and neural tissue, but still allows the independent manipulation of the different parts of the early embryo by loss and gain of function techniques (Fig. 1, B, C). We used this assay to investigate the influence of mesodermal *Hox* expression on *Hox* expression in the neural tissue.

In wraps containing a combination of wild type SO, NOM and AC tissue, *Hoxd1* is expressed in the NOM mesoderm derivatives and in the neural tissue of the animal cap. A wrap containing exclusively NOM tissue only expresses *Hoxd1* in the NOM but does not do so in the animal cap.

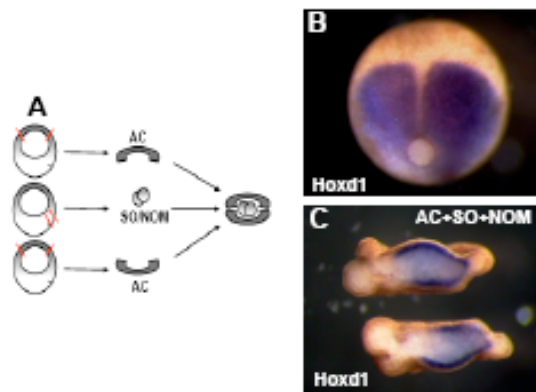


Figure 1: the wrap assay mimics signals within the embryo. **A-** a piece of NOM and a piece of SO are combined within a sandwich of 2 animal caps. **B-** *Hoxd1* expression in stage 12.5 embryo. Note that the SO area does not show expression. **C-** combination of NOM+SO leads to the expression of *Hoxd1* in the neurectoderm mimicking the embryo situation.

We have previously demonstrated that the requirement of the organizer for ectodermal *Hox* expression in the wrap combinations is due to its function in inducing neural tissue in the animal caps (Wacker *et al.*, 2004a). The absence of *Hox* expression in the animal caps of wraps lacking organizer tissue is likely due to a general lack of *Hox* expression in the early embryonic epidermis. Interestingly however, a combination of two SO explants within a single wrap is in itself not sufficient to induce *Hoxd1* expression, despite neuralization of the animal cap. These observations demonstrate a requirement for a non organizer mesoderm derived signal for the induction of *Hoxd1* expression in the animal cap (Wacker *et al.*, 2004a).

We exploited the possibilities for loss and gain of function in the wrap assay to investigate whether *Hoxd1* itself is part of this signal. We performed loss and gain of function by injecting either morpholino antisense oligonucleotides or capped messenger RNA in *Xenopus laevis* blastomere stage embryos and used grafts from these embryos in different wrap combinations. NOM from morphan embryos was explanted and combined in a wrap assay with organizer mesoderm and animal caps from uninjected control embryos (Fig. 1, A). In these wraps, knocking down *Hoxd1* in the NOM prevents its expression in the animal cap (Fig. 2, D), showing that *Hoxd1* expression in the mesoderm is required for *Hoxd1* expression in the neurectoderm.

Conversely we asked whether *Hoxd1* expression itself is sufficient to provide the signal for inducing *Hoxd1* expression in the neurectoderm. We took SO grafts from embryos injected with *Hoxd1* mRNA. These embryos thus contain *Hoxd1* messenger and protein in the Spemann organizer in which *Hoxd1* is normally not expressed.

In the wrap assay, we combined an explant of SO expressing *Hoxd1* with a wild type SO graft, to exclude that the treatment affects a necessary SO function. We subsequently monitored *Hoxd1* expression with a probe recognising the endogenous *Hoxd1* 3' UTR to prevent over-staining and masking of the endogenous animal cap signal due to the high abundance of the injected *Hoxd1* message in the organizer mesoderm (Fig. 2, B, C). In contrast to the control wraps containing wild type Spemann organizer tissue only, we observe *Hoxd1* expression in the overlying animal cap tissues of the wraps including organizer tissue ectopically expressing *Hoxd1*. Our results show that in the wrap assay, *Hoxd1* expression in the NOM is necessary and sufficient to induce *Hoxd1* expression in the neurectoderm through vertical signaling.

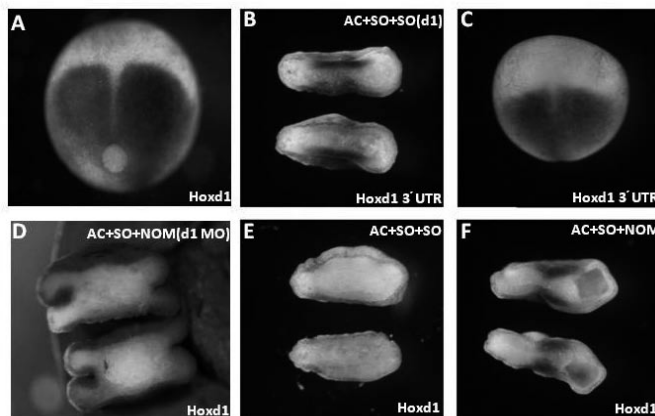


Figure2: *Hox* expression in the mesoderm is necessary and sufficient to its expression in the neurectoderm. **A-***Hoxd1* expression at stage 12.5. **B-**wrap with SO+SO ectopically expressing *Hoxd1* show endogenous *Hoxd1* expression in the neurectoderm. **C-***Hoxd1* 3' UTR expression. **D-**Knocking *Hoxd1* in the NOM abolishes *Hoxd1* expression in the neurectoderm. **E-**A wrap with 2 SO does not show any *Hoxd1* expression in the caps. **F-** control wrap: only the combination SO+NOM triggers *Hoxd1* expression in the caps.

Vertical signalling and *Hox* gene transfer

We have shown that *Hox* gene expression in the mesoderm is required for the vertical posteriorizing signal derived from the non organizer mesoderm. It is however not clear what constitutes the signalling agent itself (i.e. the actual molecule that travels between mesoderm and neurectoderm).

It could be that this is a *Hox* gene induced classical signal transduction factor, like retinoic acid, Wnt or FGF. Another possibility is that the HOX proteins themselves constitute at least part of the signal. It has been shown that several homeoproteins like for instance HOXA-5 (Chatelin *et al.*, 1996) and Engrailed (Joliot *et al.*, 1998) are internalized by cells and are able to translocate from cell to cell. The capacity of these different proteins to translocate between cells resides in a sequence in the third helix of the homeodomain (known as penetratin), which is also present in *Xenopus laevis* HOXD1 (Fig. 3; reviewed in Prochiantz, 2005; Joliot and Prochiantz, 2004).

To investigate whether the *Xenopus laevis* HOXD1 protein possesses the translocation properties expected on basis of its penetratin sequence, we constructed a green fluorescent proteins (GFP) fusion with the *Xenopus laevis* HOXD1 homeodomain (d1HD-wt-GFP). We produced recombinant d1HD-wt-GFP and GFP proteins using HIS-tag based nickel purification. We assayed the transfer properties of these recombinant proteins in *Drosophila* imaginal discs. These imaginal discs consist of a heterogenous cell population and are epithelial structures (Royet, 1998) and a very important feature

Penetratin	RQIKIWFQNRRMKWKK
Xl Hoxd1 homeodomain wt ²⁰⁰PSEYGVTSPPCNVRTNFTTKQLTELEKEFHFNKYLTRARRIEIANSLQLNDTQVKIWFQNNRMKQKKRE²⁵⁹	
Xl mut Hoxd1 homeodomain ²⁰⁰PSEYGVTSPPCNVRTNFTTKQLTELEKEFHFNKYLTRARRIEIANSLQLNDTQVKISRQNNRMKQKKRE²⁵⁹	

Figure3: Penetratin peptide and HOXd1 homeodomain. *Xenopus laevis* Hoxd1 exhibits a penetratin like peptide in its homeodomain. Amino-acids in red have been reported to be necessary for the transfer ability. In green are highlighted the mutant amino-acids in the penetratin like sequence.

is that, in contrast to the yolky *Xenopus* embryos, they are transparent and therefore easy to assay by fluorescence microscopy. We incubated imaginal discs in PBS with GFP recombinant protein (Fig. 4, A) and d1HD-wt-GFP recombinant protein (Fig.4, B)

for 15min at room temperature. The discs were washed with PBS three times, and presence of GFP protein was monitored by fluorescence microscopy.

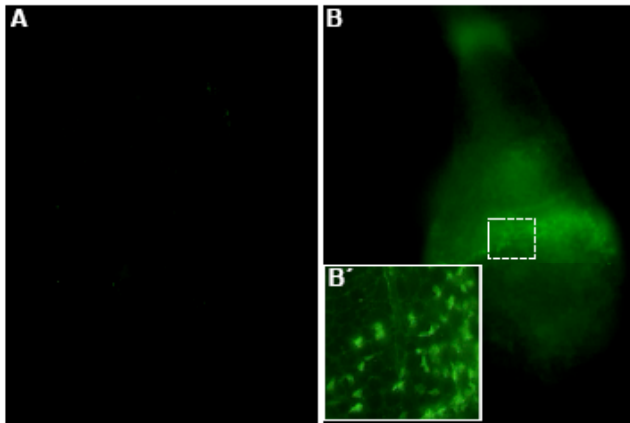


Figure 4: GFP linked to *Hoxd1* homeodomain efficiently enters cells of the wing imaginal disc. **A**-imaginal disc incubated with GFP. **B**-imaginal disc incubated with GFP linked to *Hoxd1* homeodomain (d1HD-wt-GFP). **B'**-close up of the plan in B. d1HD-wt-GFP enters efficiently the cells.

GFP recombinant protein was efficiently washed out and was not present in the preparations and had apparently not entered the cells. In contrast, d1HD-wt-GFP recombinant protein however efficiently crossed cell membranes and entered the cells (Fig. 4, B'). As control we constructed a mutated HOXD1 GFP fusion construct (mut-d1HD-GFP) expected to exhibit no intercellular transfer. It has been reported that two amino acids **WF** within the penetratin like sequence are required for the translocation of homeoproteins (Fig. 3; Brunet *et al.*, 2005). In the mut-d1HD-GFP we mutated the **WF** sequence into **SR**. Imaginal discs were incubated in PBS with d1HD-GFP-wt or mut-d1HD-GFP recombinant proteins (Fig. 5). As expected the mut-d1HD-GFP is not internalized into the cells and behaves as wild type GFP.

These data show that the HOXD1 homeodomain sequence possesses the expected translocation properties and that the transfer is dependent on a classical penetratin sequence in the homeodomain.

A phenotypic assay demonstrates functionality of homeodomain transfer in the *Xenopus* embryo

The HOXD1 homeodomain sequence is thus efficiently internalized as shown by the experiments in the *Drosophila* imaginal disks. These results however do not reveal whether this process is active as well in the *Xenopus laevis* early embryo and importantly, whether the internalized protein is also transported to the right subcellular compartments for proper functioning. We investigated whether a recombinant HOXD1 protein exhibits biological functionality when applied in the extracellular space of *Xenopus* embryos.

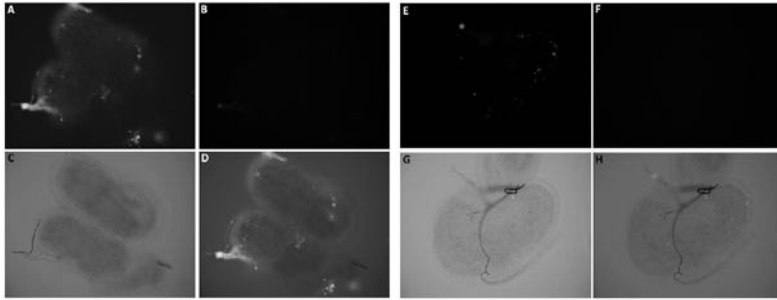
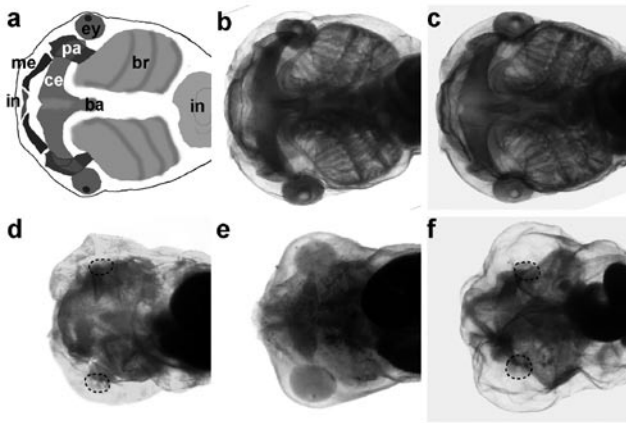


Figure 5: Mutation of two amino acids WF into SR within the penetratin like sequence abolishes the translocation from medium into cells. **A-D**, incubated imaginal discs with d1HD-GFP-wt in the medium. **A**, green channel. **B**, red channel. **C**, bright field. **D**, merge. **E-H**, incubated imaginal discs with mut-d1HD-GFP in the medium. **E**, green channel. **F**, red channel. **G**, bright field. **H**, merge.

When *Hoxd1* is overexpressed according to standard methods by injection of an mRNA at the two cell stage, the craniofacial structures are strongly perturbed and show a severe reduction of size of the branchial arches (Fig. 6, C, D-E). This phenotype provides a method for experimentally assessing HOXD1 functionality. A wild type recombinant HOXD1 protein was produced and injected either in the blastomeres of an early embryo (Fig., D), or into the extracellular space of the blastocoel during gastrulation (Fig., E). Stage 45 tadpoles were stained for cartilage with Alcian blue, to reveal the craniofacial structures (McNulty *et al.*, 2005). Upon injection of HOXD1 recombinant protein, craniofacial structures are reduced similarly as in mRNA injections, without a difference between intra- or extracellular injection conditions. Injection of GFP into the blastocoel during early gastrulation does not result in any phenotype (Fig. 6, compare A to B) showing that the blastocoels injection on its own does not have toxic side effects.

These experiments show that the recombinant protein induces the same effect when applied intra- or extracellularly and demonstrate that the HOXD1 protein is internalized by the embryo and afterwards does not differ in function from intercellular applied HOXD1.

Figure 6: Craniofacial structures of *Xenopus laevis* tadpoles upon injection of *Hoxd1* mRNA or HOXD1. a- Schematic ventral view of a uninjected untreated control embryo. b-uninjected embryo. c-Embryo injected with recombinant GFP into the blastoecel at blastula stage. d-Embryo injected with *Hoxd1* mRNA at 4 cell stage. e- Embryo injected with recombinant HOXD1 protein into 4 cell stage embryo. f- injection of recombinant HOXD1 protein into the blastoecel. This leads to similar phenotypes as in d or f. Infrastrale (in), Meckel's cartilage (me), palatoquadrate (pa), ceratohyale (ce), basibranchiale (ba), branchial arches (br), eye (ey), intestine (in)



Discussion

Proper patterning the neural tissue is a very crucial event during development for proper locomotion and behaviour of the animal later on. Several studies have tried to reveal the nature of molecules involved in neural patterning. The Spemann organizer has proven to be one major source of patterning and neutralizing molecules (Stern, 2000). Previous work brought evidence that signals acting within the plane of the neural tissue (called planar signals) are necessary but not sufficient to account for the complicated A-P pattern of the neural plate (Grunz *et al.*, 1998; Chen *et al.*, 2000). Emerging evidence suggests the involvement of signals from the non-organizer mesoderm in several vertebrates (Stern *et al.*, 2006; Wacker *et al.*, 2004a).

Hox transcription factors are key players in determining positional information along the anteroposterior axis of the vertebrate embryo. In *Xenopus laevis*, it has been reported that *Hox* gene expression is initiated in the mesoderm of the gastrula and is then subsequently found in the adjacent neurectodermal cells (Wacker *et al.*, 2004a). We investigated the existence of a potential link between *Hox* mesodermal expression and its subsequent expression in the neurectoderm using the wrap assay (Jansen *et al.*, 2007).

We show a requirement for a vertical, non organizer mesodermal derived signal for the induction of neural *Hox* expression. In addition it turns out that the *Hox* expression itself is part of the information necessary for this signal and that there thus is a direct connection between the early mesodermal and early neurectodermal *Hox* codes. We have so far not identified the actual signaling agent travelling between the mesoderm and neurectoderm and the possibilities exist that this is a *Hox* induced downstream signal transduction factor or a *Hox* protein itself.

The *Drosophila* *Hox* protein, Antennapedia, has been shown to exhibit the capacity to travel across biological membranes by means of a 'penetratin' sequence within the homeodomain (Joliot *et al.*, 1991). Due to the extreme conservation of *Hox* proteins throughout evolution, the emerging idea is that the presence and the conservation of sequences allowing homeoprotein internalization and secretion might be of physiological relevance (reviewed in Prochiantz, 2005; 2003). We thus decided to investigate if *Xenopus* HOX proteins (HOXD1 being first expressed) exhibit a capacity to translocate across biological membranes, and might act as a vertical signaling molecule. We conclusively demonstrate that HOXD1 exhibits properties of transfer and that the transfer is active in the *Xenopus* gastrula and does not negatively affect the functionality of the protein. This now opens the way for further research to investigate this process further out in the embryo. A crucial experiment that should be performed is to block this signal by injection of specific antibodies against *Hox* proteins which in case of extracellular transport are expected to block the transfer process.

The simplicity of a HOX protein transfer, between mesoderm and neurectoderm would elegantly solve the question of molecules involved in the vertical signalling, because there is a one on one connection between the information from the mesoderm and the neurectoderm. The biological relevance for the phenomenon of homeoproteins transfer has long been unclear until recently. Brunet and co-workers have shown that Engrailed-2 protein functions as an extracellular neuron guidance molecule by entering axonal projections and locally altering levels of translation of target molecules (Brunet *et al.*, 2005).

In summary, our data indicate that in *Xenopus* a causal connection between its initial mesodermal expression and its later neural expression of a *Hox* gene. The HOX protein encoded by this gene also shows the unconventional transfer behaviour reported for members of other *Hox* paralogous groups in *in vitro* studies (Amstetten *et al.*, 2003).

A recombinant HOXD1 protein seems to retain its biological activity after being internalized within the cells of the embryo as has been reported by *in vitro* studies (Nedelec *et al.*, 2004). The ability of other *Xenopus* HOX proteins to activate direct target genes is currently under investigations.

Material and methods

Injection of morpholinos and mRNA

Embryos were staged according to (Nieuwkoop and Faber 1956). *In vitro* fertilization, embryo culture, and mRNA injection, were carried out as previously described (Jansen *et al.*, 2007). *Hoxd1* and *Hoxd1* anti-sense morpholino oligonucleotide (MO, Gene-Tools Inc.) were previously described (McNulty *et al.*, 2005). Wrap assays were made as previously described (Jansen *et al.*, 2007).

Detection of gene expression by *in situ* hybridization

Whole mount *in situ* hybridization was performed as previously described (Harland, 1991), except that the RNase step was omitted.

Recombinant proteins synthesis

Preparation of His-tagged recombinant proteins were as described (Cao *et al.*, 2004). XIHoxd1 OFR (McNulty *et al.*, 2005) was amplified by the use of the following primers: **F**-CGGGATCCATGAATTCCTACCTAGAATA; and **R**-CCCAAGCTTCTAGGGTGAAGCGTCCTTGGA Primers for the synthesis of cargo protein Hoxd1 homeodomain (wild type or mutant) linked to the green fluorescent protein (GFP) are as follows:

F – GGGATCCATGCCCTGCAATGTGAGGACAAA; R –
GGAATTCATTCCACCCCCTCCGCCACCCCTTTCCCTTTTCTTTTGTTC for the
unmutated homeodomain (d1HD-GFP-wt); and for the mutated homeodomain, the primers
are: F – GAAGATCTCTCGACAGAACAGAAGAATGAAACAAA; R –
CCCAAGCTTCTAGGGTGAAGCGTCCTTGA

Imaginal discs preparation

Drosophila melanogaster larvae were dissected in PBS. Imaginal discs were put in another dish containing PBS + recombinant proteins. The discs were extensively washed with PBS after 15 min of incubation at room temperature.

Acknowledgments

We are very grateful to Petra Pandur for providing *Drosophila* larvae. A special thank to Ovidiu Sirbu for discussions. Finally a special thank to Joost Woltering for encouragements and extensive editorial comments.

