Vertical Signalling Involves Transmission of Hox Information from Gastrula Mesoderm to Neurectoderm

Nabila Bardine1*, Gerda Lamers1, Stephan Wacker2, Cornelia Donow2, Walter Knoechel2, Antony Durston1*

1. Leiden University, Institute of Biology, Sylvius Laboratory, Leiden, The Netherlands, 2. University of Ulm, Institute of Biochemistry, Ulm, Germany

* A.J.Durston@gmail.com (AJD); bardine.nabila@gmail.com (NB)

Abstract

Development and patterning of neural tissue in the vertebrate embryo involves a set of molecules and processes whose relationships are not fully understood. Classical embryology revealed a remarkable phenomenon known as vertical signalling, a gastrulation stage mechanism that copies anterior-posterior positional information from mesoderm to prospective neural tissue. Vertical signalling mediates unambiguous copying of complex information from one tissue layer to another. In this study, we report an investigation of this process in recombinates of mesoderm and ectoderm from gastrulae of *Xenopus laevis*. Our results show that copying of positional information involves non cell autonomous autoregulation of particular Hox genes whose expression is copied from mesoderm to neurectoderm in the gastrula. Furthermore, this information sharing mechanism involves unconventional translocation of the homeoproteins themselves. This conserved primitive mechanism has been known for three decades but has only recently been put into any developmental context. It provides a simple, robust way to pattern the neurectoderm using the Hox pattern already present in the mesoderm during gastrulation. We suggest that this mechanism was selected during evolution to enable unambiguous copying of rather complex information from cell to cell and that it is a key part of the original ancestral mechanism mediating axial patterning by the highly conserved Hox genes.
Introduction

Determination of regional specificity along the anterior-posterior (A-P) axis of the vertebrate *Xenopus laevis* and of all other vertebrates begins during gastrulation. This patterning involves interactions between the Spemann organizer (SO) and surrounding tissues that are key events leading to genesis of the basic body plan [1]. Classical embryology also revealed a remarkable phenomenon known as vertical signalling, a mechanism that copies A-P positional information from non-organiser mesoderm (NOM) to overlying neurectoderm during gastrulation [2]. Nieuwkoop showed that the A-P pattern of the amphibian embryo is generated in the developing nervous system (neurectoderm) during gastrulation by two types of signals: activation and transformation [3]. These signals are emitted by mesoderm and act on ectoderm. The SO secretes activation signals that induce neuralisation of the ectoderm but induce only an anterior neural identity (presumptive forebrain) [4]. Tissue recombination and grafting experiments indicated that this patterning occurs similarly in an amniote (the chick embryo) as in the anamniote Amphibia [5] and also that transformation signals originate from NOM (lateral and paraxial mesoderm) and that they induce a progressively more caudal identity of the neural tissue [5–11]. Known signalling pathways have been proposed to be involved in transformation: these include retinoids [11–13], FGFs [14] and Wnts [15]. Elevated concentrations of these signalling molecules cause posteriorisation by inducing relatively posterior positional values in the neurectoderm and each of these factors has been proposed to act as a posterior to anterior gradient within the embryo [8–13]. These factors possibly mediate some of the known planar signals that act along the A-P axis of the germ layers during gastrulation. However, planar signals do not fully account for the properties of neural transformation. The use of exogastrulae and other approaches revealed a second type of signal. It appeared that posterior neural markers were expressed only at the border between ectoderm and mesoderm in exogastrulae, excluding the existence of very extensive planar signalling [16]. These and other observations rule out that axial patterning is fully accounted for by planar signals and the evidence actually indicates that the second type of signalling: vertical signalling is the more important in generating the A-P pattern of the neural plate [16–19]. The nature of the molecules involved in vertical signalling remains unclear.

We reported previously that expression of the A-P determining Hox genes begins during gastrulation in *Xenopus laevis*. This initial expression starts in NOM tissue in the mid-gastrula [20]. It presumably corresponds with the initial “Hox induction field” or “opening zone” that has been reported in other vertebrates [21, 22]. This is the place where Hox codes are first available and later, when these mesodermal cells involute and come to lie underneath prospective neural tissue, the same A-P information spreads to that neural tissue by the end of gastrulation [20]. Interestingly, use of a tissue recombination assay, the wrap assay showed a requirement for NOM and SO to induce Hox gene expression in the neurectoderm [23]. The SO induces the embryonic ectoderm to a neurectodermal identity and the NOM induces expression of various Hox genes in the neuralised
ectoderm ([23], reviewed in [24]). These steps appear to correspond to Nieuwkoop’s activation and transformation steps, respectively. In this study, we investigated the importance of the Hox genes for vertical signalling using the wrap assay to provide a controlled setting for investigating signalling from mesoderm to neurectoderm. We found that Hox expressing NOM mesoderm provides positional information for the adjacent neurectoderm. Moreover, in these wrap assays, mesodermal expression of each of the Hox genes that we investigated appears to be necessary as well as sufficient for inducing the expression of the same gene in the neurectoderm. The absolute requirement for mesodermal expression of the homologous Hox gene is striking. It indicates an extraordinarily high degree of specificity for vertical signalling: a feature that was already anticipated from the embryological data [2]. Using recombinant Hox proteins, we also detected transfer of the homologous Hox proteins between NOM mesoderm and neurectoderm during this non cell autonomous Hox autoregulation. We suggest that this is the basis of the extraordinary specificity of this mechanism. We also detected Xenopus Hox protein uptake from the medium by Xenopus embryonic cells as well as Drosophila imaginal discs. Considering that Hox codes are thought to be synonymous with A-P positional information and that the NOM induces neurectodermal Hox gene expression, our data suggest that expression of individual Hox genes is copied specifically from NOM to neurectoderm during neural transformation. This information sharing transfer involves a peculiar protein transfer of the homeoproteins themselves as has previously been described by others [25]. This primitive mechanism has presumably been conserved in evolution to enable specific information sharing between tissue layers in a very simple and direct manner.

Results
Hox expression in the mesoderm is necessary and sufficient to induce neurectodermal Hox expression

We have shown previously that zygotic Hox gene expression is first initiated in the non-organizer mesoderm (NOM) in the Xenopus mid gastrula (St. 10.5) [20]. This expression then spreads during gastrulation to the overlying prospective neurectoderm that was induced from embryonic ectoderm by signals from the Spemann organizer (SO). We investigated whether Hox expression in the gastrula’s NOM causes Hox expression in the neurectoderm. This was done using a recombinant wrap assay, in which pieces of SO and NOM mesoderm were combined with two animal caps (Fig. 1A). The main advantage of this assay is that it mimics the embryonic situation with respect to proximity and physical connectivity of SO, NOM and neural tissue, but still allows the independent manipulation of different parts of the early embryonic tissues by loss and gain of function techniques. It also offers the advantage of clear consistent tissue separation [20, 23] (Fig. 1A). Interestingly, a combination of two SO explants within a single wrap is in itself not sufficient to induce Hox gene expression,
despite causing neuralization of the animal cap (Fig. 1Ac, 1Bb, 1Cb). These results show that this assay mimics the embryonic situation (Fig. 1A, B, C) and they demonstrate a requirement for a NOM derived signal for induction of Hox expression in the clearly neuralised animal cap. We used loss of function via morpholino anti-sense nucleotides to investigate whether a single Hox gene knockdown in NOM affects neurectodermal expression of the same gene. MO’s for each of Hoxd1, Hoxb4 and Hoxb9 respectively were injected into zygotes and the ability of NOM from the injected embryos to induce neurectodermal expression of these Hox genes in wraps was examined in comparison to NOM loaded with control morpholino (ctMO). Fig. 1 shows that NOM knockdown of each of these Hox genes prevented expression of the same gene in the neurectoderm (Fig. 1Ae, Bd, Cd) whereas ctMO loaded NOM did not impair neurectodermal Hox expression in vivo. Conversely we asked whether ectopic expression of a single Hox gene in
mesoderm can induce its own expression in the neurectoderm. In this gain of function approach we took SO grafts from embryos zygotically injected with a single Hox mRNA. An explant of SO expressing this single Hox gene was then combined in a wrap with a wild type SO graft and 2 animal caps. The untreated SO was included to exclude that Hox ectopic expression blocks a necessary SO function. When Hoxd1, Hoxb4 or Hoxc6 respectively were ectopically expressed in SO, each efficiently induced its own expression in neurectoderm in such a wrap recombinant (Fig. 2B’, C and D). We detected induced expression of the endogenous Hox gene using a 3’ UTR probe that does not recognize the ectopically expressed messenger. These results show that ectopic expression of a single Hox gene in SO induces expression of the same gene in neurectoderm.

We also show in Fig. 2 that these wrap explants show the normal expression of tissue layer markers as seen in a normal gastrula (Fig. 2A, A’, A”, A”’, E and F). This combinatorial tissue assay shows no intermingling between the different mesoderm types or between mesoderm and ectoderm during wrap culture and the markers and lineage labelling clearly show different localisations of mesoderm and neurectoderm within the wrap (See Fig. 2 legend for further description). There is clear correspondence of Hox expression with expression of both mesodermal and neurectodermal markers.

Altogether, our results show that in the wrap setting, a single Hox gene expressed in the mesoderm is necessary and sufficient to induce its own expression in overlying neurectoderm.

Vertical signalling and Hox protein transfer

Tissue recombination as in the wraps used in this study and in grafting experiments in various vertebrates have shown consistently that mesodermal signals induce the neural A-P pattern (see introduction, [3,5,6]). Major signalling pathways, including Wnts, Retinoids and Fgfs have been suspected to have a role [11–15]. Another type of molecule that may be involved in vertical signalling is the Hox proteins themselves. This idea was inspired by results from Prochiantz and colleagues who showed that homeoproteins move from cell to cell due to the existence of a special sequence, penetratin, within the homeodomain [25–27]. This led us to investigate whether Xenopus Hoxd1 protein possesses such a sequence. Fig. 3A shows that Xenopus laevis Hoxd1 protein has a penetratin sequence in its homeodomain. We constructed a labelled (myc tagged) Hoxd1 and analysed its ability to translocate from mesoderm to neurectoderm. Myc-Hoxd1 was ectopically expressed in SO as in S1 Figure. The labelled (myc tagged) Hoxd1 protein was seen to translocate from the labelled SO mesoderm to unlabelled mesoderm and neurectodermal tissues in these wrap recombinates. Hox proteins translocate due to their penetratin sequence, which is known to confer transfer capacity and cargo functions that depend on key amino-acids in the penetratin sequence (see Fig. 3A). Mutation of these amino acids abolishes the protein’s ability to translocate (Fig. 3A, WF to SR). Chimeric GFP constructs were constructed containing either a wild type translocatable Hoxd1 homeodomain...
(d1-HD-gfp), or a homeodomain containing a mutated penetratin sequence that blocks translocation (mut d1-HD-gfp) and translocation of these constructs in wraps was compared to that of wild type GFP as a control. Fig. 3B–D” shows that whereas wild type GFP and mut d1-HD-gfp both stay localised within the mesoderm in the wrap assay, the d1-HD-gfp spreads to the edges of the explant (Fig. 3C–C”). Early incubation times (0–50 min) already show spreading of the signal in an open wrap. S1 Figure also shows that the myc-tagged Hoxd1 protein travels to the edges of the recombinate, far from its initial site of expression (see
S1A and B). These results clearly show that *Xenopus Hoxd1* contains a functional penetratin sequence with a cargo function as has been reported for other homeoproteins.

Another striking property of the penetratin sequence is its ability to translocate from cell to cell non species specifically. We tested whether the *Xenopus Hoxd1* homeodomain shows this property. *Drosophila* imaginal dics were incubated with...
wild type GFP, d1-HD-gfp or mut d1-HD-gfp recombinant proteins for 15 min at room temperature. **Fig. 4** shows clearly that d1-HD-gfp (**Fig. 4B, B’**) is taken up by the discs while GFP alone (**Fig. 4A**) or mut d1-HD-gfp (**Fig. 4C**) do not cross the epithelial layer of the disc. This is in accordance with previous reports that showed that mutation of 2 key amino acids within the penetratin sequence abolishes the uptake of the latter [26]. This result shows a clear non species specific mechanism. This is a very robust mechanism that could allow massive information transfer between mesoderm and neur ectoderm during neural transformation. This lack of species specificity is consistent with homeoprotein transfer (see discussion) but we cannot rule out from the data that a highly conserved ligand-receptor mechanism (like BMP- chordin) is involved.

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

The ability of *Xenopus* Hoxd1 homeoprotein to exhibit properties predicted by its penetratin sequence is interesting. However, this unconventional transfer was not shown to account for an embryonic function. So, we investigated whether recombinant Hoxd1 protein exhibits biological activity when applied in the extracellular space of *Xenopus* gastrula embryos. When Hoxd1 is overexpressed according to standard methods by injecting messenger RNA, the embryo’s craniofacial structures are strongly affected and show a severe reduction of size of the branchial arches as we have previously reported (**Fig. 5d**) [28]. This reflects posteriorisation of A-P positional information in the head region. A wild type recombinant Hoxd1 protein was produced and injected either into the blastomeres of an early embryo (**Fig. 5e**) or into the extracellular space of the blastocoel during gastrulation (**Fig. 5f**). Upon injection of Hoxd1 recombinant protein, craniofacial structures are reduced similarly as following standard mRNA injections, without any difference between the intra- and extracellular injection conditions. Conversely injection of GFP protein into the blastocoel during early gastrulation causes no phenotype (**Fig. 5, compare b to c**) excluding a possible toxic side effect due to blastoecel injections per se. These results show that the
recombinant protein induces the same effect when applied intra- or extracellularly and demonstrate that the Hoxd1 protein is efficiently internalized by the embryo and that it seems to retain its function.

Altogether, these results indicate a role of the conserved Hox transcription factors in signalling during neural transformation, and in vertical signalling.

**Discussion**

Axial patterning of the central nervous system is a crucial event during development. It enables proper locomotion and behaviour of the animal. Many studies have investigated the nature of the molecules involved in neural patterning. The Spemann organizer is one major source of patterning and neuralizing molecules [1, 29]. Previous work also 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 [16–19]. Emerging evidence has also indicated the involvement of signals from the non organizer mesoderm (NOM) in several vertebrates including the frog [4–9]. These clearly mediate what has been called vertical signalling [2]. We have investigated
the involvement of Hox genes in vertical signalling. Hox transcription factors are the key players in determining positional information along the anteroposterior axis of the vertebrate embryo. In *Xenopus laevis*, it has been shown that Hox gene expression is initiated in the NOM of the gastrula and then subsequently spreads to the adjacent neurectodermal cells [20]. We investigated the possibility of a link between mesodermal Hox expression and the subsequent Hox expression in the neurectoderm using a tissue recombination assay (wrap assay), that was previously proven to be an ideal way to manipulate different tissues in a controlled manner [20, 23]. We previously showed a clear requirement for a vertical, non organizer mesoderm (NOM) derived signal for the induction of neural Hox expression. Here, we show that the NOM Hox expression itself is part of the information necessary for this signal and that there is thus a clear connection between the early mesodermal and early neurectodermal Hox codes. Our investigations using gain and loss of function approaches revealed that this requirement for NOM Hox expression is specific. It is striking that the loss of function MO approach shows that each Hox gene we tested actually needs to be translated itself in NOM to enable its own induced expression in neurectoderm. This implies that mesodermal function of each of these Hox genes is uniquely required for its own induction in neurectoderm in the wrap recombinates used for this test. No other Hox gene or developmental regulator that is co-expressed in the recombinate can substitute this function. This is high specificity. We have so far not identified the actual signalling agents travelling between the mesoderm and neurectoderm and the possibilities exist that these are Hox induced downstream signal molecules or the Hox proteins themselves. However, it would be very difficult to account for the very high degree of specificity we see by a conventional mechanism, such as a morphogen gradient. In previous studies, the *Drosophila* Hox protein *Antennapedia* and other homeoproteins have been shown to exhibit the capacity to travel across biological membranes by means of a ‘penetratin’ sequence within the homeodomain under conditions excluding any classical endocytosis mechanism [25–27]. This is a much more specific mechanism and due to the extreme conservation of Hox proteins throughout evolution, it is likely that the presence and the conservation of sequences allowing homeoprotein internalization and secretion might be of physiological relevance during development. We thus decided to investigate if a *Xenopus* Hox protein (Hoxd1: the first expressed Hox gene) exhibits a capacity to translocate across biological membranes. We conclusively demonstrated that Hoxd1 exhibits properties of transfer and that the transfer is active in the *Xenopus* gastrula and it does not negatively affect the functionality of the protein. This is a very specific mechanism that delivers a very specific signal, a fact that is entirely consistent with our finding that induction of the expression of a particular Hox gene in neurectoderm specifically requires expression of this same Hox gene itself (and not just, for example of one of its paralogues) in NOM mesoderm. Other studies point to unexpectedly specific actions of secreted homeoproteins in brain function [43, 44].
Gradients of FGFs, RA and Wnts have all been proposed to be involved in activation of Hox gene expression along the anterior-posterior axis [12–15]. Biologically active retinoids are good candidates for signalling from mesoderm to neurectoderm because they are synthesized in mesoderm by RALDH and destroyed there by Cyp 26, and it seems that retinoid receptors are present mainly in the neurectoderm [31–34]. The synthetic and degradative regions of the mesodermal axis frame the hindbrain: the axial segment where Hox paralogue groups 1–5 have their anterior neurectodermal boundaries and show collinearly graded RA sensitivities [31, 33]. In Xenopus, disruption of RA production in the mesoderm has no effect on Hox mesodermal expression while it only downregulates Hox in the neurectoderm [34]. However, Hox paralogue groups 6–13 do not exhibit RA sensitivity [31]. FGFs and Cdx might play a role in regulating neurectodermal Hox expression here [35, 36]. We think that these different pathways work in concert with specific Hox signals. In fact, it has been shown in the chordate Amphioxus that the labial Hox gene (Hox1) mediates (all of) the effects of RA on the hindbrain (including regulating expression of more posterior Hox genes) [37]. This fits the finding that knocking out the whole Hox1 paralogue group in Xenopus knocks out expression of more posterior Hox genes as well as Hox1 [28]. It has also been shown previously that homeoprotein transfer and conventional signalling pathways work in concert in other contexts [29, 30].

The growing evidence that homeoproteins are signalling molecules could be the key to understanding the whole picture of neural patterning. Indeed, the simplicity of Hox protein transfer would elegantly solve the patterning problem because it accounts for the one to one connection between mesodermal and neurectodermal Hox expression. Hox homeoprotein transfer was discovered almost 30 years ago [25, 26] but its biological relevance has been unclear until recently [38, 39, 43, 44]. We suspect that homeoprotein transfer is an ancient conserved signalling mechanism that is central to the ancestral Hox patterning mechanism that evolved in bilateria. Findings by Prochiantz and colleagues indicate that homeoprotein transfer is an extremely ancient form of signalling that antedates the division between animals and plants and presumably thus evolved before specific ligand-receptor mediated signalling. We suspect it has been selected and retained for vertical signalling because it is so eminently suitable to mediate Hox information copying.

In summary, our data show that at least several Hox genes expressed in the Xenopus gastrula show a causal connection between initial mesodermal expression and later neural expression. This connection is highly specific. The Hox protein encoded by at least one of these genes also shows unconventional intercellular transfer behaviour during vertical signalling and seems able to exert its biological function after passing through cell membranes as reported for members of other Hox paralogous groups in former studies.
Summarising Conclusions

1. We show, in Figs. 1 and 2, that Hox proteins expressed in *Xenopus* gastrula NOM mesoderm induce their own expression in gastrula neur ectoderm. There is thus non cell autonomous autoregulation of individual Hox genes. We equate this autoregulation with the classical concept of ‘vertical signalling’: copying of positional information from gastrula mesoderm to gastrula neur ectoderm. Loss of function experiments for three different Hox genes, using morpholinos (Fig. 1), show that this homologous induction is specific for each of these three genes. Loss of function for each particular gene in NOM mesoderm deletes its own expression in neur ectoderm. Clearly, no other endogenously expressed Hox gene or developmental regulator can substitute for this unique function. Fig. 2 shows that Hox expression induced in Hox gain of function experiments is localised not only in the (SO) mesoderm in which the Hox gene was introduced into a wrap recombinant but also widely in mesoderm and in neur ectoderm. See Figs. 1 and 2 for details.

2. The specificity of vertical signalling led us to consider an unorthodox mechanism. Prochiantz and colleagues have shown that Hox proteins and other homeoproteins can transfer from cell to cell. They transfer by virtue of a special ‘penetratin’ sequence within the homeobox of the homeoprotein which can be mutated to ablate transfer of the homeoprotein (internalisation or secretion). This mechanism is now well known and well accepted and has been shown to mediate several different developmental functions. It is exactly the type of mechanism needed to mediate ‘vertical signalling’, which is copying of positional information from one germ layer to another and which we have shown (above) to be mediated by copying of Hox gene expression from one germ layer to another.

3. We examined whether Prochiantz transfer occurs during vertical signalling between mesoderm and neur ectoderm from *Xenopus* gastrulae. Myc tagged Hoxd1 was introduced into wrap recombinates in NOM mesoderm and cultured over 6–8 hrs. Over this time course, Myc tagged Hoxd1 spreads to every part of the recombinate, from the SO mesoderm in which it was introduced; to neur ectoderm as well as mesoderm. We conclude that Hoxd1myc is transferred from cell to cell. (See S1 Figure). We also tested the dependence of this transfer on ‘penetratin’. We thus constructed a chimeric protein consisting of Hoxd1 homeodomain (including the penetratin sequence) coupled to *GFP*. We tested the mobility of this labelled protein in comparison to a mutant where two essential amino acids in the penetratin sequence are mutated and the capacity for transfer is ablated; and with GFP itself. The wild type Hoxd1HD-GFP (*d1-HD-gfp*) was transferred from mesoderm to neur ectoderm (transfer made more visible using red phalloidin background staining). The mutated homeodomain coupled to GFP protein (*mut d1-HD-gfp*) and GFP were not. We conclude that the homeodomain of the Hox gene Hoxd1 transfers from mesoderm to neur ectoderm (Fig. 3). And
that Hoxd1 itself is indeed transferred from gastrula mesoderm to gastrula neurectoderm by the Prochiantz mechanism (S1 Figure).

4. We tested the species specificity of d1-HD-gfp homeoprotein transfer. This was taken up by Drosophila imaginal discs whereas GFP and mut-d1-HD-GFP were not. This finding is consistent with the known extreme aspecificity of Prochiantz signalling (which occurs both in animals and plants and presumably evolved very early on, possibly before ligand-receptor signalling). This emphasizes that Hoxd1 passes cell membranes in a species independent manner, confirming the previous indications of species independent Hox transfer and excluding a requirement for species specific forms of ligand-receptor signalling (Fig. 4).

5. We tested the functionality of Hoxd1 protein transfer. When this protein was injected into the blastocoel of Xenopus gastrulae, it gave the same posteriorising phenotype as cytoplasmically injected Hoxd1 mRNA or protein. This again emphasizes that Hoxd1 protein passes cell membranes and that the transported protein is functional.

6. Finally, the findings above demonstrate unambiguously that non cell autonomous autoregulation of Hox proteins mediates vertical signalling/neural transformation in the Xenopus gastrula and that at least for Hoxd1, a mechanism involved is Prochiantz intercellular homeoprotein transfer.

Methods Summary

Ethics

The Leiden University Animal Experimentation Ethics (DEC) Committee have approved this work.

Handling and treating Xenopus embryos

Embryos were staged according to Nieuwkoop and Faber [40]. In vitro fertilization, embryo culture, mRNA and antisense morpholino oligonucleotides (MO, Gene-Tools Inc.) and protein injections were used as previously described [41]. In situ hybridization analyses were carried out as formerly reported [41]. The wrap assay was as described previously [41]. Microsurgery was carried out using hair knives. Mesodermal tissue (NOM or SO) was explanted and the epithelial layer removed. After keeping these tissue explants for a few minutes in MBS, they were placed between two animal caps, which had been cut immediately before to prevent curling. Wraps were cultivated in 1%MBS for about 30 min and then transferred to 0.1% MBS. Wraps were usually fixed 6 to 8 h after preparing them, when control embryos reached stage 14–15 (i.e. mid neurula).
Preparation of recombinant GFP proteins

Preparation of recombinant GFP proteins were as described [42]. Primers for the synthesis of cargo proteins Hoxd1 homeodomain (wild type and mutant) linked to GFP are as follows: Forward – GGGATCCATGCCCTGCAATGTGAGGACAAA; and Reverse – GGAATTCATTCCACCCCCCTCAGCCACCCCTTTCACCTTTTCTTTTTGTTTCA for the wild type Hoxd1 homeodomain (d1-HD-gfp); and for the mutated Hoxd1 homeodomain (mut d1-HD-gfp), the primers are: Forward - GAAGATCTCTCGACAGAACAGAAGAATGAAACAAAA; and Reverse - CCAAGCTTCTAGGGTTGAGGCCTCTCTTGA. MO’s sequences are: Hoxd1 MO [28]; Hoxb9 MO: 5’ – TGACCGACCCACCAGCTCTCCCAC – 3’; and the standard control morpholino (ctMO) from Genes-Tools Inc.

Immunolocalisation

Immunolocalisation was performed using monoclonal anti-myc tag antibody (Sigma-Aldrich), and a secondary anti-mouse Alexa-488 antibody (Invitrogen) according to a previously described protocol [34]. Wraps were made as described earlier [23]. Wraps were bisected prior to cryosectioning (5 μm) and processed for in situ or immunolocalisation, and in situ was performed before being embedded in gelatin and sectioned (10 μm).

Phalloidin counterstaining

Wraps were bisected prior to phalloidin (Invitrogen) step because the uptake by the cells was greatly improved.

Drosophila Imaginal Discs

Drosophila larvae were dissected in PBS. Imaginal discs were incubated in PBS + recombinant proteins at room temperature for 15 min. The discs were then extensively washed with PBS prior to analysis. Each experiment is based on 20 or more discs.

Numbers

In each of the experiments in Figs. 1–5, S1 and S2 Figures, each photo is representative of 20 to more recombinates or embryos or imaginal discs, all of which gave the same result. See figure legends for details.

Supporting Information

S1 Figure. Immunolocalisation of Hoxd1-myc in wraps. Immunolocalisation of Myc-labelled Hoxd1 protein after 6–8 hrs of culture in a wrap. A: control wrap. B: immunolocalisation of myc-tagged Hoxd1 in a wrap containing [AC(SO+d1-myc)+SO]AC] shows spreading of myc-Hoxd1 protein from mesoderm to the
outer layers of the wrap (neurectoderm) while no signal is detected in the control wrap (A). The signal spreads throughout the recombinate to originally unlabelled neurectoderm as well as originally unlabelled mesoderm. Each photo represents 10 wraps giving identical results.

doi:10.1371/journal.pone.0115208.s001 (TIF)

S2 Figure. Open wrap reveals an early spreading of the cargo d1-HD-GFP. A: Left, a wrap containing [AC(SO+mut-d1-HD-gfp)SO]AC]. The upper animal cap is replaced by a small glass coated with BSA. This allows us to follow changes of the signal in time. Right, a wrap containing [AC(SO+d1-HD-gfp)SO]AC]. B: after 30 min of incubation, both wraps stayed open. C: after 50 min of incubation, the signal in the wrap with d1-HD-gfp shows a little spreading towards the outside of the SO. This shows that, even after such a short incubation time, the penetratin is capable of playing its cargo function for the GFP protein. Each photo represents 10 wraps giving the same result.

doi:10.1371/journal.pone.0115208.s002 (TIF)

Author Contributions

Conceived and designed the experiments: AJD NB SW CD. Performed the experiments: NB GL CD SW. Analyzed the data: AJD NB. Contributed reagents/materials/analysis tools: GL CD SW WK. Contributed to the writing of the manuscript: AJD NB.

References

1. Spemann H, Mangold H (1924) Induction of embryonic primordial by implantation of organizers from a different species. In: Foundations of Experimental Embryology (eds. Willier B.H. and Oppenheimer J.M.), 144–184. New York: Hafner.

2. Mangold O (1933) Uber die Induktionsfahigkeit der verschiedenen Bezirke der Neurula von Urodelen. Naturwissenschaften 21: 761–766.

3. Nieuwkoop PD (1952) Activation and organisation of the central nervous system in amphibians, III. Synthesis of a new working hypothesis. J Exp Zool 120: 83–108.

4. De Robertis EM, Kuroda H (2004) Dorsal-ventral patterning and neural induction in Xenopus embryos. Annu Rev Cell Dev Biol 20: 285–308.

5. Stern CD (2005) Neural induction: old problem, new findings, yet more questions. Development 132(9): 2007–2021.

6. Stern CD, Charite J, Deschamps J, Duboule D, Durston AJ, et al. (2006) Head-tail patterning of the vertebrate embryo: one, or many unresolved problems. Int J Dev Biol 50: 3–15.

7. Gould A, Itasaki N, Krumlauf R (1998) Initiation of rhombomeric Hoxb4 expression requires induction by somites and a retinoic pathway. Neuron 21: 39–51.

8. Muhr J, Jessell TM, Edlund T (1997) Assignment of early caudal identity to neural plate cells by a signal from caudal paraxial mesoderm. Neuron 19(3): 487–502.

9. Woo K, Fraser SE (1997) Specification of the zebrafish nervous system by nonaxial signals. Science 277(5323): 254–257.

10. Fuji H, Nagai T, Shirasawa H, Doi JY, Nishimatsu S, et al. (2002) Anteroposterior patterning in Xenopus embryos: egg fragment assay system reveals a synergy of dorsalizing and posteriorizing embryonic domains. Dev Biol 252(2): 15–39.
11. Kolm PJ, Sive H (1997) Retinoids and posterior neural induction: a reevaluation of Nieuwkoop’s two-step hypothesis. Cold Spring Harb Symp Quant Biol 62: 511–521.

12. Durston A, Timmermans J, Hage W, Hendriks H, de Vries H, et al. (1989) Retinoic acid causes an anteroposterior transformation in the developing central nervous system. Nature, 340, 140–4

13. Godsave SF, Koster CH, Getahun A, Mathu M, Hooiveld M, et al. (1998) Graded retinoid responses in the developing hindbrain. Dev Dyn 213(1): 39–49.

14. Kengaku M, Okamoto H (1995) bFGF as a possible morphogen for the anteroposterior axis of the central nervous system in Xenopus. Development 121(9): 3121–30.

15. Kiecker C, Niehrs C (2001) A morphogen gradient of Wnt/beta-catenin signalling regulates anteroposterior neural patterning in Xenopus. Development 128(21): 4189–4201.

16. Chen Y, Holleman T, Pieler T, Grunz H (2000) Planar signalling is not sufficient to generate a specific anterior/posterior neural pattern in pseudoexogastrula explants from Xenopus and Triturus. Mech Dev 90(1): 53–63.

17. Poznaski A, Keller R (1997) The role of planar and early vertical signaling in patterning the expression of Hoxb-1 in Xenopus. Dev Biol 184(2): 351–366.

18. Grunz H, Schüren C, Richter K (1995) The role of vertical and planar signals during the early steps of neural induction. Int J Dev Biol 39(3): 539–43.

19. Ruiz i Altaba A (1992) Planar and vertical signals in the induction and patterning of Xenopus nervous system. Development 116(1): 67–80.

20. Wacker SA, Jansen HJ, McNulty CL, Houtzager E, Durston AJ (2004) Timed interactions between the Hox expressing non-organizer mesoderm and the Spemann organizer generate positional information during vertebrate gastrulation. Dev Biol 268: 207–219.

21. Joubin K, Stern CD (2001) Formation and maintenance of the organizer among the vertebrates. Int J Dev Biol 45(1): 165–75.

22. Deschamps J, van Den Akker E, Forlani S, De Graaff W, Oosterveen T, et al. (1999) Initiation, establishment and maintenance of Hox gene expression patterns in the mouse. Int J Dev Biol 43: 635–650.

23. Jansen HJ, Wacker SA, Bardine N, Durston AJ (2007) The role of Spemann organizer in anterior-posterior patterning of the trunk. Mech Dev 124(9–10): 668–81.

24. Harland R, Gerhart J (1997) Formation and function of Spemann’s organizer. Annu Rev Cell Dev Biol 13: 611–67.

25. Chatelin J, Volovitch M, Joliot AH, Perez F, Prochiantz A (1996) Transcription factor hoxa-5 is taken up by cells in culture and conveyed to their nuclei. Mech Dev 55(2): 111–7.

26. Joliot A, Maizel A, Rosenberg D, Trembleau A, Dupas S, et al. (1998) Identification of a signal sequence necessary for the unconventional secretion of Engrailed homeoprotein. Curr Biol 8(15): 856–63.

27. Joliot A, Prochiantz A (2004) Transduction peptides: from technology to physiology. Nature Cell Biol 6(3): 189–196.

28. McNulty CL, Peres JN, Bardine N, van den Akker WM, Durston AJ (2005). Knockdown of the complete Hox paralogous group1 leads to dramatic hindbrain and neural crest defects. Development 132(12): 2861–1871.

29. Layalle S, Volovitch M, Mugat B, Bonneaud N, Parmentier ML, et al. (2011) Engrailed homeoprotein acts as a signaling molecule in the developing fly. Development Jun; 138(11): 2315–23. Doi:10. 1242/ dev.057059.

30. Wizenmann A, Brunet I, Lam JS, Sonnier L, Beurdeley M, et al. (2009) Extracellular Engrailed participates in the topographic guidance of retinal axons in vivo. Nov 12; 64(3): 355–66. Doi:10. 1016/ j.neuron.2009.09.018.

31. Godsave SF, Koster CH, Getahun A, Mathu M, Hooiveld M, et al. (1998) Graded retinoid responses in the developing hindbrain. Dev Dyn 213(1): 39–49.

32. Swindell EC, Thaller C, Sockanathan S, Petkovich M, Jessel TM, et al. (1999) Complementary domains of retinoic acid production and degradation in the early chick embryo. Dev Biol 216(1): 282–96.
33. Gavalas A, Krumlauf R (2000) Retinoid signaling and hindbrain patterning. Curr Opin Genet Dev 10(4): 380–6.

34. Lloret Vilaspasa F, Jansen HJ, de Roos K, Chandraratna RA, Zile MH, et al. (2010) Retinoid signalling is required for information transfer from mesoderm to neuroectoderm during gastrulation. Int J Dev Biol 54(4): 599–608.

35. Pownall ME, Tucker AS, Slack JM, Isaacs HV (1996) eFGF, Xcad3 and Hox genes form a molecular pathway that establishes the anteroposterior axis in Xenopus. Development 122(12): 3881–92.

36. Bel-Vialar S, Itasaki N, Krumlauf R (2002) Initiating Hox gene expression: in the early chick neural tube differential sensitivity to FGF and RA signalling subdivides the HoxB genes in two distinct groups. Development 129(22): 5103–15.

37. Schubert M, Holland ND, Laude V, Holland LZ (2006) A retinoic acid-Hox hierarchy controls both anterior/posterior patterning and neuronal specification in the developing central nervous system of the cephalochordate amphioxus. Dev Biol 296(1): 190–202.

38. Brunet I, Wein C, Piper M, Trembleau A, Volovitch M, et al. (2005) The transcription factor Engrailed-2 guides retinal axons Nature 438(7064): 94–8.

39. Di Lullo E, Haton C, Le Poupon C, Volovitch M, Joliot A, et al. (2011) Paracrine Pax6 activity regulates oligodendrocyte precursor cell migration in the chick embryonic neural tube. Development 138(22): 4991–5001.

40. Nieuwkoop PD, Faber J (1956) Normal table of Xenopus laevis (Daudin). Amsterdam: North-Holland Publishing Company.

41. Bardine N, Donow C, Korte B, Durston AJ, Knöchel, et al. (2009) Two Hoxc6 transcripts are differentially expressed and regulate primary neurogenesis in Xenopus laevis. Dev Dyn 238(3): 755–65.

42. Cao Y, Knöchel S, Donow C, Miethe J, Kaufmann E, et al. (2004) The POU factor Oct-25 regulates the Xvent-2B gene and counteracts terminal differentiation in Xenopus embryos. J Biol Chem 279(42): 43735–43743.

43. Spatazza J, Lee HHC, Di Nardo AA, Tibaldi L, Joliot A, et al. (2013). Choroid plexus-derived Otx2 homeprotein contrains adult cortical plasticity. Cell Rep. Jun 27; 3(6): 1815–1823. Doi:10. 1016/j.celrep.2013.05.014.

44. Sugiyama S, Di Nardo AA, Aizawa S, Matsuo I, Volovitch M, et al. (2008). Cell 134, 508–520, August 8. Doi:10.1016/j.cell.2008.05.054.