# Coordination of *Hox* identity between germ layers along the anterior-to-posterior axis of the vertebrate embryo

Ferran Lloret Vilaspasa


PhD Developmental Biology
Department of Anatomy and Developmental Biology
University College of London (UCL)
London, United Kingdom
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‘ I, Ferran Lloret Vilaspasa confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. '

Thesis

for the obtainment of a PhD in Development Biology at the University College of London under the supervision of Prof. dr. Claudio D. Stern and Prof. dr. Antony J. Durston (Leiden University). To be defended

by

Ferran Lloret Vilaspasa

Born in Barcelona, 05-08-1977

A la meva família...

## Abstract

During early embryonic development, a relatively undifferentiated mass of cells is shaped into a complex and morphologically differentiated embryo. This is achieved by a series of coordinated cell movements that end up in the formation of the three germ layers of most metazoans and the establishment of the body plan. *Hox* genes are among the main determinants in this process and they have a prominent role in granting identity to different regions of the embryo. The particular arrangement of their expression domains in early development corresponds to and characterises several future structures of the older embryo and adult animal.

Getting to know the molecular and cellular phenomena underlying the correct *Hox* pattern will help us understand how the complexity of a fully-formed organism can arise from its raw materials, a relatively undifferentiated fertilised egg cell (zygote) and a large but apparently limited repertoire of molecular agents.

In the present work I have concentrated on the specific factors, and their mechanism of action, that set up the *Hox* expression patterns in the gastrula and neurula embryo. I have put special emphasis on the initiation of *Hox* expression, which takes place first in the non-organiser mesoderm and subsequently in the neuroectoderm. I investigated the role of retinoid signalling and found that it is required during late gastrulation for activation of 3' *Hox* genes in the neuroectoderm. Furthermore, I show evidence that the earliest phase of expression in the gastrula mesoderm requires Wnt, but not retinoid, activity. Moreover, the most 3' *Hox* genes are direct targets of the Wnt pathway, whereas other *Hox* genes are indirectly regulated. Finally, I provide preliminary results that suggest a potential mechanism for communication between non-organiser mesoderm and neuroectoderm mediated by HOX protein intercellular signalling. This phenomenon would enable a direct coordination of *Hox* pattern between the two tissues.

## Contents

## Abbreviations

| | |
|---|---|
| A-P axis | Anterior-to-posterior axis |
| bFGF | Basic fibroblast growth factor |
| BMP | Bone morphogenetic protein |
| BSA | Bovine serum albumine |
| cAMP | Cyclic adenosine monophosphate |
| CNS | Central nervous system |
| CRABP | Cellular retinoic acid binding protein |
| CSLM | Confocal laser scanning microscopy |
| CYP26 | Cytochrome p-26 |
| DMSO | Dimethyl sulfoxide |
| DTT | Dithiothreitol tetraacetate |
| ER | Endoplasmic reticulum |
| EMT | Epithelial to mesenchymal transition |
| En2 | Engrailed 2 protein |
| ErbB | Epidermal growth factor receptor |
| ERNI | Early response to neural induction |
| FGF | Fibroblast growth factor |
| FGFR | Fibroblast growth factor receptor |
| FITC | Fluorescein isothiocyanate |
| GDF | Growth differentiation factor |
| hCG | Human chorionic gonadotropin |
| MBT | Mid-blastula transition |
| MHB | Mid- hindbrain boundary |
| NIC | Non-injected control |
| PCP | Planar-cell-polarity |
| PDGFA | Platelet-derived growth factor A |
| Pg | Paralogous group |
| PKC | Protein kinase C |
| PCR | Polymerase chain reaction |
| r | Rhombomere |
| RA | Retinoic acid |
| RALDH2/Raldh2 | Retinaldehyde dehydrogenase 2 |
| RAR | Retinoic acid receptor |
| RAREs | Retinoic acid responsive elements |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| RXR | Retinoid receptor |
| TGF-β | Transforming growth factor beta |
| TRITC | Tetramethylrhodamine isothiocyanate |
| UTR | Untranslated regions |
| VAD | Vitamin A-deficient |
| vHnf1 | Hepatocyte nuclear factor 1-beta |
| Wnt | Wingless/Int |
| YSL | Yolk-syncytial layer |

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**Chapter 1**

## General introduction

During embryogenesis it is both common and essential that neighbouring cells but also tissues communicate with one another. There are several mechanisms to fulfil such requirement (e.g. via gap junctions, cell-to-cell adhesions or free diffusible molecules), though in the end they all provide with cooperation, be it physical or molecular.

Within the early embryo in particular, sensing and cross-talking with the immediate environment is the rule, for a relatively undifferentiated mass of cells called blastula needs to rearrange itself into a complex and morphologically differentiated embryo. The process known as gastrulation is the landmark through which the three germ layers of most metazoans (ectoderm, mesoderm and endoderm) are formed. As a result of these drastic series of cell movements, the main axes of the embryo (dorsal-ventral and anterior-posterior, in vertebrates) soon become apparent. While this general reorganisation lays down the rudiments of the body plan, further characterisation of the three nascent layers is already taking place. This includes patterning, by endowing them with regionally distinct molecular identities. Thus, for example, the future head starts to express a combination of genes that is not to be found anywhere else along the anterior-posterior (A-P) axis.

Gastrulation and the subsequent stages of development must consequently be a period of high demand on cell-to-cell and tissue interactions, whereby

coordination of the extensive changes it brings about at the morphological and molecular levels can be achieved. I have focused the present project on a series of events framed within this developmental period, starting from the beginning of gastrulation and continuing way into neurulation (period of morphological characterisation of the fundamental plan of the nervous system). These events, albeit having induction of *Hox* genes in common, are in principle distinct phenomena. However, in agreement with the highly dynamic scenario of cell movements and coordination that comprises them, they are likely to be interconnected. In fact, the final experiments described in this thesis explore a possible mechanism whereby the two other events could be interrelated.

**Gastrulation**

Gastrulation literally means the formation of the belly. However, during this time the three germ layers (two in diploblastic organisms) emerge and the main embryonic axes are laid down. It is a fundamental process, common to all metazoans (Haeckel, 1874; reviewed in Schierenberg, 2005), which nevertheless presents as a large variety of mechanisms across the animal kingdom. Terms like ingression, epiboly, invagination, involution or delamination, define different patterns of cell movements often encountered in embryonic development. A given combination of these phenomena accounts for gastrulation in a given organism. Considering the types of cell movements is very important for understanding how these developmental events occur, and their molecular controls. Cell-to-cell and tissue interactions not only underlie but also are dependent on cell movements.

Here, an overview of chick and *Xenopus* gastrulation is presented. These are the two model organisms used in the present work. Having a comprehensive knowledge of the features common to both species and the peculiarities inherent to each one of them, may allow us to establish a better comparison.

*Xenopus laevis* (clawed African frog)

The early *Xenopus laevis* embryo is a yolky cell mass with high molecular heterogeneity that progressively divides into more and more cell entities as cleavage goes on (Nieuwkoop, 1989). During the first 10 to 12 rounds of division, which are synchronous and occur without significant increase of the embryo's mass, quick cell division cycles provide continuous replication of DNA without a chance for transcription of new (zygotic) mRNA; only at the end of this period, coinciding with the start of asynchronous divisions, do new transcripts an tRNAs begin to be produced (the so-called mid-blastula transition (MBT)) (Newport and Kirschner, 1982a,b). By the late blastula stage, the embryo is a morphologically undifferentiated ball made up of thousands of cells. Different regions are however clearly distinguishable, based on yolk and pigment distribution: a dark and yolk-poor animal pole on top and a light-coloured yolk-rich vegetal pole deposited at the bottom. The animal pole consists of a thin epithelial layer (animal cap) concealing a large cavity called the blastocoel immediately underneath; the latter internally separates the animal cap from the yolky cells in the vegetal pole (Nieuwkoop, 1989). Forming the side wall of the blastocoel all around the blastula, a transitional area called the marginal zone separates vegetal and animal cap cells in the periphery (Nieuwkoop, 1989).

Furthermore, a subtle distinction already defines the future dorsal side of the embryo. Briefly after fertilisation, a cortical rotation process translocates the most superficial region of the egg towards the sperm entry point, approximately 30° relative to the deeper cytoplasm (Gerhart, 1987). This results in a rearrangement of pigment and other maternal components, which can be observed opposite to the sperm entry point as a semicircular greyish extension (grey crescent) of the darker pigmented area in a vegetal direction (Vincent et al., 1986; Nieuwkoop, 1989). The grey crescent indicates the dorsal side of the future embryo (Vincent et al., 1986).

Gastrulation in *Xenopus laevis* starts when a mass of cells internalises on the dorsal side of the marginal zone of the blastula embryo. These are called bottle cells and are named because of the shape they acquire. They arise at the antipodes of the sperm entry point, in the middle of the grey crescent (reviewed in Nieuwkoop, 1989). A local invagination is formed as their internal side swells up and their superficial side becomes narrow (Keller, 1981). From there on, a further and more generalised internalisation takes place, as the bottle cells push the deeper cells inwards. The latter are located ahead of the former and will become head mesendoderm (prechordal plate) (reviewed in Keller, 1981). A process of involution then takes place which brings more and more superficial cells towards the inside of the embryo; these cells form a cohesive layer that crawls under the surface, in the animal direction, to form the archenteron roof (reviewed in Keller, 1981). Initial invagination of the bottle cells causes the appearance of the dorsal blastopore lip, the site whereby the organiser internalises; as the process of invagination advances, the lip expands towards the lateral

marginal zone and eventually forms a complete a circle, closing up at the ventral marginal zone (reviewed in Keller, 1981). Cells internalised through the blastopore lip give rise to mesoderm and endoderm, whereas those remaining on the surface of the embryo become ectoderm (Nieuwkoop, 1989).

Involution at the most dorsal side brings the organiser cells straight through to the blastocoel (the animal pole cavity of the blastula), which shrinks upon being invaded (Nieuwkoop, 1989). Around mid-gastrulation, this organiser mesoderm thins out and spreads along the blastocoel roof (Keller, 1980; Keller et al., 1992). In doing so, it lays down the rudiments of the A-P axis as the prospective notochord and prechordal plate (axial structures) (Keller, 1980; Keller, 1992). Moreover, it remains in close apposition to the overlying neuroectoderm (still separated by a thin gap called Brachet's cleft), which enables easy communication between the two tissues (Wacker et al., 2000). As gastrulation proceeds and bottle cells are being formed throughout the blastopore, involution brings lateral and ventral marginal zone up to underlie the animal cap. Such mesoderm gives rise to paraxial, lateral and ventral mesoderm (reviewed in Nieuwkoop, 1989). As on the axial (dorsal) side of the embryo, this tissue comes into apposition with the overlying ectoderm (neuroectoderm for the most dorso-lateral positions, i.e. paraxial mesoderm), which again facilitates communication (e.g. eventually between somites and neural plate) (Nieuwkoop, 1989). The endoderm, which originates in the most vegetal yolky cells of the embryo, has migrated more or less passively along with the mesoderm (Winklbauer and Schürfeld,

1999) and, being the deepest layer, it becomes the lining of a new cavity called the archenteron (primitive gut) (reviewed in Keller, 1981).

A phenomenon called convergent extension is largely responsible for driving involution during gastrulation (Keller, 1980) This phenomenon involves coordinated cell rearrangements, combining two components: cell intercalation, either within (medio-lateral intercalation) or across layers of the tissue, and elongation along the A-P axis, first of the non-involuted marginal zone and later of the involuting mesendoderm (Keller, 1980). The contribution of each component differs depending on the localisation of the tissue across and along the marginal zone (Keller, 1980); as a result, each area of the embryo will be furnished with the amount and the arrangement of involuted tissue necessary for the future development of the pertinent structures.

In the meantime, cells remaining on the surface of the embryo (forming ectoderm) undergo epiboly, whereby a multilayered epithelial sheet thins out into an epithelium with fewer cell layers and expands (Keller, 1980); this permits the rest of the morphogenetic movements that transform the blastula into a gastrula. Furthermore, convergence and extension plays an important role in distribution of the ectoderm, just like it does in mesoderm (described above) (Keller, 1980). Consequently, the prospective neural plate (gastrula neuroectoderm) is laid down along the midline in the dorsal surface of the embryo, stretched along the A-P axis and aligned with the underlying axial and paraxial nascent mesoderm (Keller, 1980; Wacker et al., 2000).

The molecular cues and downstream activation of intracellular cascades underlying gastrulation in *Xenopus laevis* is fairly well understood,

considering the apparent complexity of the phenomenon. Studies conducted mainly *in vitro* by modification of the substratum, drew attention to the interaction between the involuted mesoderm and the blastocoel as a main driving force for gastrulation (Winklbauer, 1990). Moreover, identification of fibronectin as an essential component in the extracellular matrix of the blastocoel paved the molecular side of the story (Winklbauer, 1990; Nagel and Winklbauer, 1991). A proper fibronectin network is a prerequisite for directional migration of the mesoderm, but it has only a permissive role (Winklbauer, 1991; Nagel and Winkelbauer, 1991). Correct deposition of fibronectin occurs during the blastula stage, where FGF and activin-like signals emanating from the contiguous nascent mesoderm may play a role (Winklbauer, 1999). Directional migration of the involuting mesoderm in a posterior-to-anterior (animal pole) sense is an intrinsic property of the most anterior mesoderm, but it also depends on external cues from the overlying blastocoel roof (Nagel and Winklbauer, 1991). Extracellular guidance may be set up by early action of activin-like and FGF signals (Winkelbauer, 1999), too. Nevertheless, it seems that PDGFA is involved as a direct guiding molecule, which correlates with its expression in the blastocoel roof and that of PDGFA-receptor (PDGFRalpha) in the involuted mesoderm (Nagel et al., 2004). Less is known about the signals driving epiboly and convergent extension in the superficial layer (prospective ectoderm) during gastrulation, presumably because it may be a rather passive phenomenon in *Xenopus laevis*, where cells keep moving and intercalating at least partly driven by the superficial tension created by active rearrangements in the marginal zone and involuting mesoderm (Keller, 1980). In any case, studies

on the cytoskeleton and surrounding extracellular matrix of the superficial layer of the gastrula, implicate both E-cadherin in maintaining the epithelial cohesion necessary for epiboly (Levine et al., 2004) and fibronectin probably to aid in resisting the superficial tension created by convergent extension (Rozario et al., 2009).

A large amount of evidence has identified different components of the non-canonical Wnt pathway with an indispensable role in convergence and extension by morpholino knock-down of endogenous: *frizzled7,* a Wnt11 receptor (Djiane et al., 2000); *Wnt11*, expressed in the organiser downstream of *Brachyury* (Tada and Smith, 2000); *Xror2*, a tyrosine kinase associated to frizzled7 (Hikasa et al., 2002); *syndecan4*, a Wnt co-receptor that interacts with both frizzled and Dishevelled to transduce the non-canonical pathway (Muñoz et al., 2006); *glypican4*, a transmembrane protein that synergises with Dishevelled activity (Ohkawara et al., 2007; Caneparo et al., 2007). Changes in the cytoskeleton that may enable mobility and particular adhesions seem to be the ultimate effect of this pathway's activity (Tanegashima et al., 2008 and references therein). On the other hand, the Wnt/β-catenin (canonical) pathway has also been shown to participate in convergent extension events, particularly by counteracting and modulating Wnt non-canonical pathway activity (Kühl et al., 2001; Caneparo et al., 2007; Tahinci et al., 2007). Convergence and extension movements also require FGFs at different levels: to activate transcription of *Brachyury* directly (Conlon and Smith, 1999) and to modify morphology of the presumptive mesodermal cells without affecting expression of *Brachyury* (Chung et al., 2005; Nutt et al., 2001). Furthermore, *Xnr3*, the only *Xenopus laevis nodal*

homologue expressed in the organiser and devoid of mesoderm-inducing capabilities appears to be essential for the *Brachyury* expression domain in the dorsal marginal zone (Yokota et al., 2003). Finally, attenuating ErbB with morpholinos has recently suggested a requirement of this membrane receptor for proper cell adhesion and motility leading to convergent extension (Nie and Chang, 2007a,b).

By the end of gastrulation (stage 13), after a process that spans about 6 hours, the involution is complete, the three germ layers have been created, and the rudiments of the A-P and dorsal-to-ventral axes have taken shape. Thus, an organised embryo with an obvious bilateral symmetry has arisen.

Chick (*Gallus gallus*)

The late blastula chick embryo (stage XIII Eyal-Giladi) appears as a flat disc made of several thousands of cells (blastoderm), protruding on the gravitational top of the yolk. It consists of a superficial epithelial layer called epiblast and a deeper (proximal to the yolk) less coherent layer called hypoblast (surrounded by the area opaca endoderm in the periphery) (Stern, 1990; Stern 2004). The disc is in turn divided into a central translucent region (area pellucida) and a marginal denser belt of cells (area opaca) (reviewed in Watt et al, 1993). The embryo proper arises exclusively from the area pellucida of the epiblast, except that primordial germinal cells, albeit originating from the epiblast, appear to colonise the hypoblast during blastula stages and later (Ginsburg and Eyal-Giladi, 1986; Karagenç et al., 1996; Tsunekawa et al., 2000). The presence of the hypoblast is crucial to ensure that only one posterior end and hence one single A-P axis is formed (Bertocchini and Stern, 2002).

Already in the early blastula (stage X Eyal-Giladi), appearance of the Koller's (Rauber's) sickle, a crescent-shaped thickening in the posterior edge of the area pellucida, reveals the first signs of polarity within the embryo (Koller, 1882; Izpisúa-Belmonte et al., 1993; Callebaut and Van Nueten, 1994), which has been predetermined in the pre-laid egg by rotation and gravity within the progenitor hen's oviduct (Kochav and Eyal-Giladi, 1971). The early blastula hypoblast (stage X Eyal-Giladi) consists of scattered clusters of cells that spread out tightly adhering to the overlying epiblast. By the late blastula stage (stage XIII Eyal-Giladi), the clusters have fused into a continuous and looser sheet that encompasses the whole area pellucida (reviewed in Stern 1990; Stern, 2004). This late hypoblast arises in a posterior-to-anterior direction and, as soon as it has been fully laid down, it is quickly displaced by the endoblast (an extraembryonic tissue originating in the deep layer at the boundary with the area opaca) (Stern, 1990)) to form the germinal crescent in the anterior side of the area opaca (Ginsburg and Eyal-Giladi, 1986).

Immediately following a brief transitional and last blastula stage (stage XIV Eyal-Giladi), another structure appears. It is the primitive streak, which seems to arise at least partly from Koller's (Rauber's) sickle (Izpisúa-Belmonte et al., 1993; Hatada and Stern, 1994; Bachavarova et al., 1998; Stern, 2004). Appearance of the streak marks the beginning of gastrulation. During gastrulation cells migrate through the primitive streak to a deeper level. These cells will give rise to endoderm and mesoderm, whereas those remaining on the surface of the embryo include progenitors of the ectoderm (Canning and Stern, 1988; Stern and Canning, 1990). The prospective

endoderm migrates deepest in respect to the original epiblast and displaces the endoblast, whereas prospective mesodermal cells come to lie between the prospective ectoderm at the surface and the proximal to the yolk endoderm (Lawson and Schoenwolf, 2003; Kimura et al., 2006). Formation of the primitive streak begins as a local accumulation of cells in the most posterior part of the epiblast (stage 2 HH) (reviewed in Chuai and Weijer, 2008). Gastrulation proceeds as the streak expands towards the anterior part of the epiblast, and by doing so delineates the A-P axis; it reaches about 3/4 of the area pellucida and there it stops growing (Psychoyos and Stern, 1996). At that point (stage 4 HH), a special structure is being formed in the most anterior tip of the streak; this is the Hensen's node, a pit whereby the future notochord and prechordal plate cells (axial structures), as well as the precursors of the floor plate, medial somites and endoderm migrate (Selleck and Stern, 1991).

In order to provide for a continuous source of ingression, streak and node recruit cells scattered all across the epiblast (Canning and Stern, 1988; Stern and Canning, 1990). Primitive streak elongation is driven by a series of cell rearrangements collectively known as Polonaise movements (Gräper, 1929; Voiculescu et al., 2007). Analysis of individual cells by 2-photon high resolution time-lapse imaging reveals medio-lateral intercalation of individual cells as they move across the one-layered epiblast towards the region where the streak will form, displaying an overall counter-clockwise pattern of movements (Voiculescu et al., 2007).

Meanwhile, cells have started to ingress through the growing streak as they undergo EMT (epithelial to mesenchymal transition), and eventually migrate

away under the epiblast as individual entities (Schoenwolf and Lawson, 2003; Voiculescu et al., 2007). Each cell's relative position along the streak roughly determines its destination (Psychoyos and Stern, 1996). Generally speaking, the more anterior a cell ingresses through the streak (i.e. closer to Hensen's node), the closer to the axial structures it will come to lie. For instance, prospective medial somite tissue arises from cells posterior but close to the node, whereas more lateral somite tissue, lateral plate and ventral mesoderm precursors depart from increasingly posterior streak positions (Selleck and Stern, 1991; Schoenwolf et al., 1992; Psychoyos and Stern, 1996; Sawada and Aoyama, 1999). On the other hand, cells fated to become notochord follow a special pattern of migration; upon ingression, they move shortly ahead of the node and then arrest their march (Schoenwolf et al., 1992). Only when the node starts regressing (stage 4+ HH), to trace back its previous trajectory, the notochord extends along the forming trunk and tail (Stern and Bellairs, 1984; Schoenwolf et al., 1992; Psychoyos and Stern, 1996) by incorporating descendants of resident stem cells in the node (Selleck and Stern, 1991).

By stage 4+ HH, most of the ingression has taken place and the embryo possesses both a clear bilateral symmetry and three distinct germ layers. However, a lot of reorganisation needs to take place, as many of the ingressed cells are still undergoing migration to their final destinations (Psychoyos and Stern, 1996).

Recently, the causal/consequent molecular changes accompanying gastrulation in the chick have begun to be elucidated. Voiculescu et al. (2007) revealed an essential role of the non-canonical Wnt pathway for

streak elongation; showing that interference with the endogenous specific members of the planar-cell-polarity (PCP) transduction pathway, converts the medial mode of gastrulation typical of amniotes (i.e. through the streak) into a radial anamniote type, without affecting mesoderm induction (Voiculescu et al., 2007). Beads soaked with FGF8 can induce the Wnt-PCP genes, as can rotation of the hypoblast, which expresses FGF8. In both mouse and chick, FGF8, acting through the FGFR1 receptor, has a further role in the EMT that accompanies ingression of cells through the streak (Ciruna et al., 1997; Sun et al., 1999; Yang et al., 2002), probably by modulating levels of E-cadherins; besides, it is required to activate transcription of *Brachyury* and *Tbx6* (Ciruna and Rossant, 2001). Once cells are recruited to the forming streak, they migrate away under the epiblast to be recruited eventually for different body structures and organs. FGFs have been suggested to act as chemotactic cues during mesoderm migration, in a model including FGF8-mediated repulsion of mesodermal cells by the streak and possible subsequent attraction of paraxial mesodermal cells to FGF4 secreted by the notochord (Yang et al., 2002). On the other hand, the non-canonical Wnt (PCP) pathway, by means of Wnt5a and Wnt11b expressed in the streak region, also seems to be involved in providing migrational signals, at least at later stages (Hardy et al., 2008; Sweetman et al., 2008). However, this pathway seems to be mainly active in the posterior part of the streak during neurulation, when it presents and interplay with the Wnt3a (canonical pathway); the latter may send a signal to mesodermal cells ensuing the anterior part of the streak to remain around the midline, while at the same time it may modulate restriction of the Wnt5/Wnt11b signals to the

posterior part of the streak where they instruct cells to migrate laterally (Sweetman et al., 2008). Furthermore, PDGFA expressed in the epiblast and its receptor located in the mesodermal cells also seem to provide chemotactic mechanism to attract cell migration, apparently by promoting N-cadherin activity (Yang et al., 2008). Interstingly, PDGF signalling is required to respond to FGF chemotaxis (Yang et al., 2008). Finally, collinear expression of *Hox* genes in the midline epiblast (see *Hox* genes in this Introduction) has been proposed to determine sorting of cells along the streak, so that those expressing *Hox* genes with lower paralogous group numbers ingress preferentially and migrate to more anterior positions (Iimura and Pourquié, 2006).

**Mesoderm and neural induction**

This work concentrates on signalling events regulating molecular determinants of A-P identities (mainly *Hox* genes). The very cells and tissues that participate in such events are either already located or on their way to settle in the dorsal-lateral side of the early vertebrate embryo. As emphasised in the beginning of the Introduction, early development must be particularly rich in cell-to-cell interactions, which means cells are continuously sensing the cues and changes produced in their environment. Because cells are most often organised into tissues, and tissues are also organised around each other, a given cell's environment consists largely of its surrounding cells in the same tissue and eventually those in the neighbouring tissues.

I consider it relevant to give a description on the tissues potentially involved in my investigation, including their conformation, but also the main inductive events that shape and endow them with intrinsic properties. A good knowledge of their respective natures could help for understanding how they respond to signals from the environment or they send signals themselves. Here, I will focus on two main tissues: dorsal-lateral (non-organiser or paraxial) mesoderm and neural tissue (neuroectoderm or neural plate); not only because they are the direct individual targets of my study (they express *Hox* genes), but also because, obeying to physical proximities and evidence from the literature, interaction between them is likely to be crucial to the questions I am trying to give an answer too.

Neural induction

In the first quarter of the last century, Hans Spemann and Hilde Mangold carried out a series of experiments that led them to the discovery of the amphibian 'organiser', published in 1924. By means of transplantation between different species of newt, they showed that the dorsal blastopore lip dissected from a donor species was able to form a secondary (ectopic) axis when implanted on the ventral side of the gastrula of a host species of similar age (Spemann and Mangold, 1924; Lenhoff, 1991; Sander and Faessler, 2001). Distinguishing between donor and host tissues revealed that the neural structures of the secondary axis are derived almost entirely from the host (Spemann and Mangold, 1924; Lenhoff, 1991; Sander and Faessler, 2001). Spemann and Mangold concluded that the dorsal blastopore lip can instruct surrounding tissue to become neural tissue. In other words, the amphibian dorsal blastopore lip (Spemann's organiser) has

the ability to cause neural induction (Spemann and Mangold, 1924; Lenhoff, 1991; Sander and Faessler, 2001). What grants this particular structure of the embryo its striking powers was to remain a long-lasting mystery.

A couple of decades later, still before the dawn of the molecular era, Pieter Nieuwkoop was studying the nature of inductive events that lead to the formation of the early neural tissue (neural ectoderm). Assisted by classical embryological methods of explanting and recombining tissues, he gave evidence for his “activation and transformation” stepwise hypothesis of neural induction (Nieuwkoop, 1952; Nieuwkoop, 1999). To support such a model, he explanted strips of gastrula ectoderm and fixed them either to the midline of a host neurula neural plate or to different positions along the earlier gastrula neuroectoderm. The flapping explants developed regional-specific neural characteristics (i.e. forebrain, midbrain, hindbrain or spinal cord), according to both the proximal distance to the midline and the relative position along the host neural plate where each flap was implanted (Nieuwkoop, 1952; Nieuwkoop, 1999). This and other similar experiments led him and his collaborators to propose that a signal emanating from the archenteron roof (involuted endo-mesoderm) induces the early gastrula ectoderm to become neural as soon as the two tissues contact each other (activation step); subsequently, the same involuted tissue (archenteron roof) would deliver an additional signal to instruct the activated (neuralised) ectoderm to acquire differential regional characters (i.e. midbrain, hindbrain and spinal cord) (transformation step) (Nieuwkoop, 1952; Nieuwkoop, 1999; Eyal-Giladi, 1954; Sala, 1955). Nieuwkoop’s hypothesis had coincidental points with the one proposed by T. Yamada in 1950, on two different agents

each responsible for either inducing or patterning the neural tissue (i.e. one acting inductively and one morphogenetically) (reviewed in Gerhart, 1999). These models were even preceded by a similar proposal on two gradients by F.E. Lehmann in 1942 and the experiments suggesting two different inducers to account for formation of head and trunk/tail neural structures by Holtfreter and Chuang in the 1930s (reviewed in Gerhart, 1999). However, Nieuwkoop's hypothesis put an emphasis on a timed rather than spatial coordination (segregation) of the two phenomena (activation and transformation). This view was to be supported later on by his and B. Albers's demonstration of two distinct competence periods. Early to mid-gastrula ectoderm on the one hand, and late gastrula to mid-neurula neural prospective prosencephalic explants on the other, from one newt donor species (*Ambystoma mexicanum*) implanted into another newt host species (*Triturus alpestris*) neurula neural plate, indicated that the donor's tissue age, but not the host's, determined the viability of the induction: up to about stage 12, ectoderm was sensitive (competent) to activation signals (Albers, 1987; Nieuwkoop and Albers, 1990), whereas from stages 11/12 to 16 anterior neural tissue was responsive to posteriorising (transforming) signals (Nieuwkoop and Albers, 1990).

The nature of these signals however, was not to be unravelled, and then only partially, until relatively recent times. Prior to these recent findings however, Nieuwkoop's collaborators in the Hubrecht Laboratorium led by Tony Durston (Utrecht) hinted on the intracellular molecular changes underlying neural induction, when they unveiled a significant increase in levels of both PKC (protein kinase C) (Otte et al., 1988; Otte et al., 1990)

and adenylate cyclase activities (rise in [cAMP]) (Otte et al., 1988; Otte et al., 1989), coinciding with *in vivo* neural differentiation events. PKC activation in particular was sufficient to trigger neural differentiation in non-induced ectoderm, although integrity of the two transduction pathways is required (Otte et al., 1988; Otte et al., 1989). Later on, they went on to show that differential distribution of PKC isozymes in dorsal versus ventral ectoderm corresponds to different sensitivities of these tissues to ectopic activation (Otte et al., 1992), suggesting that the more bond to activation dorsal ectoderm is therefore more competent to neuralising signals. Taking the issue further, A. Otte and R. Moon studied this differential distribution and overexpressed two PKC isozymes; they observed that the alpha isoform is preferentially localised in the dorsal ectoderm and can ectopically elevate competence in the ventral ectoderm, whereas the beta isoform is homogeneously distributed and ectopically increases propensity to differentiation, but not due to competence, in both tissues (Otte and Moon, 1992). Localised competence in the dorsal ectoderm seems to be granted by the presence of dorsal mesoderm (Otte et al., 1989; Otte et al., 1992b).

Identification of the inducing signals came with the isolation and cloning of factors specific to the Spemann's organiser. The first breakthrough came from the discovery of *noggin* (Smith and Harland, 1992), a secreted factor capable of directly (independent of mesoderm formation) inducing neural tissue upon overexpression on the ventral side of the *Xenopus laevis* embryos (Lamb et al., 1993). Next came *follistatin*, a direct neural inducer that acts by inhibiting activin-like signals (Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1994), and the isolation *chordin*, which like

*noggin* promotes the appearance of complete ectopic axes (Sasai et al., 1994). Very soon, two simultaneous papers in *Nature* shed a light on the organiser activities that provide for neural induction. Both studies suggested that signals secreted from the organiser and *BMP*s expressed in complementary domains in the non-organiser mesoderm and the ectoderm, antagonise one another to promote either neural or epidermal ectoderm, respectively (Wilson and Hemmati-Brivanlou, 1995; Sasai et al., 1995). Furthermore, one of these studies demonstrated that Chordin fulfils the characteristics of an endogenous organiser-secreted neural inducer (Sasai et al., 1995). Both *noggin* (Zimmerman et al., 1996) and *follistatin* (Fainsod et al., 1997; Iemura et al., 1998) were shown to be able to induce neural tissue by antagonising BMP activity. In addition, the three organiser-secreted factors (Noggin, Follistatin and Chordin) appeared to cause such antagonism by directly binding to BMP proteins (Zimmerman et al., 1996; Iemura et al., 1998; Piccolo et al., 1996) and thereby blocking transduction of this signal into cells.

The sudden inflow of data, seven decades after the discovery of the organiser and four since Nieuwkoop's hypothesis of neural induction, began to provide a molecular mechanism to explain the outcome of the old experiments. The so-called "default model" proposed a scenario where secreted factors from the organiser (e.g. Noggin, Follistatin, Chordin) spread and bind to an inhibitor (e.g. BMP proteins), which otherwise diverts the fate of non-induced ectoderm; this tissue, without the presence of its inhibitor, follows its natural fate, which is neural (Hemmati-Brivanlou and Melton, 1997; Weinstein and Hemmati-Brivanlou, 1997).

In the meantime, FGFs were also implicated in neural induction, with some studies suggesting that FGFs are sufficient for the process (Kengaku and Okamoto, 1993; Lamb and Harland, 1995). Blocking FGF signalling by means of a truncated receptor, demonstrated the requirement of this pathway for neural induction (Kinoshita et al., 1995), even when the induction was triggered by endogenous factors like noggin or dorsal mesoderm (Launay et al., 1996).

All these experiments, from the discovery of the first neural inducing factor (noggin) (Smith and Harland, 1992) to the latest appearance of FGF signalling as a complete stranger in the interplay between BMP and its direct antagonists (Kengaku and Okamoto, 1993; Lamb and Harland, 1995; Kinoshita et al., 1995; Launay et al., 1996), were carried out in very similar conditions: *Xenopus laevis* animal cap explants, in most instances from gastrula "non-induced" ectoderm. There was a call for an external referee, partly to have a better overview and find possible fundamental features common to the vertebrate embryo, but also to integrate FGF and BMP signals accounting for neural induction.

The amniote embryo brought fresh air into the field. Both *BMP4* and *BMP7*, as well as *FGF3*, were found to be expressed in the epiblast of chick embryos in cells fated to become neural (Streit et al., 1998; Wilson et al., 2000). By means of incubating chick epiblast explants to either soluble BMPs or FGFs, or a combination of these, Thomas Edlund's group concluded that FGF overimposes neural fate by inhibiting BMP signalling, and suggested that this fate is already determined *in utero*, that is long before gastrulation (Wilson et al., 2000). Claudio Stern's group used an *in*

*vivo* approach in which the organiser is implanted into the area opaca region, a neutral environment for testing neural induction (Storey et al., 1991; Streit et al., 1998; Streit et al., 2000). Manipulation of FGF signalling, a molecular screen for new genes activated at different times following a graft of the organiser and analysis of their expression patterns, led them to the conclusion that neural induction begins prior to gastrulation in an FGF-dependent manner (Streit et al., 2000). In a subsequent paper, Edlund's group showed that explants extracted from *in utero* embryos were already specified as neural at about stage XIII (very early blastula) (Wilson et al., 2001); moreover, neural induction by FGF was dependent on the local status (presence) of Wnt signalling in the epiblast, with cells expressing or exposed to WNTs taking on an epidermal fate and those unexposed being sensitive to FGFs induction of neural fate (Wilson et al., 2001). However, it must be taken into account that all of the explant experiments from the Edlund group use medium containing the supplement N2, which contains neuralising proteins like insulin as well as transferrin and other factors and is therefore not a "neutral" milieu for testing specification (see Linker et al., 2009).

The avian embryo helped to integrate FGF and BMP signalling and to bring in an additional role of WNTs. However, it raised the dilemma as to the existence of a model that could unify the anamniote and the amniote view. Namely, a "default model" provided by the anamniote embryo where ectodermal cells were 'per definition' fated as neural, simply didn't go along with the more regulative model derived from evidence in the chick embryo.

Even though the two models have not yet been reconciled efforts are already being done to present a unique model that accounts for vertebrate neural induction. A study by Claudio Stern's group using both chick and *Xenopus laevis* embryos as experimental organisms, makes some important points (Linker and Stern, 2004): first, *BMP4* ectopic expression in the chick prospective neural plate turns off the definitive neural marker *Sox2* but not the preneural marker *Sox3* (early *Sox3* expression); second, a preneural (e.g. expressing *Sox3*) stage of determination can be activated on epiblast cells upon FGF (WNT-free) exposure in their grafting assay, but not by several BMP inhibitors; third, definitive neural character is not achieved by combining any of the aforementioned factors, and the critical cue remains unidentified; finally, overexpression of *Smad6* (a potent BMP inhibitor) in the A4 blastomere (prospective epidermal ectoderm) doesn't induce any of the neural markers as assessed by *Sox3* and *NCAM*. In conclusion, they propose that BMP inhibition is only required as a late step in neural induction and a yet unknown factor is necessary to trigger definitive neural character (Linker and Stern, 2004). Recently, work from another group also suggested that neural induction in *Xenopus laevis* starts before gastrulation, and that this step is indeed FGF-dependent; only later is BMP inhibition required (Delaune et al., 2005). Like in the previous study by Claudio Stern's group and, as opposed to the earlier work based on animal cap explants, Delaune et al manipulate these pathways in the whole-embryo context (Delaune et al., 2005). There is however one discrepancy between in the conclusions from these two studies; whereas the former suggests that FGF activation and subsequent BMP inhibition can only turn on preneural but not definitive

neural (*Sox2*) markers, the latter study reports activation of the definitive neural tissue under the same conditions (also assessed by *Sox2* expression). The most obvious explanation for this different outcome is that the first work performs injections at the 32-cell stage (Linker and Stern, 2004) and the latter at the 16-cell stage (Delaune et al., 2005); this could imply differences in competence of the targeted cells, or more likely the regions targeted by the injection (if in physical interaction with the neural plate).

Essentially, the latest work seems to point towards a unifying model for neural induction, which is common to both anamniotes (as known for amphibians) and amniotes (from chick). This would refuse the 'per definition' neural fate of ectodermal cells and involve early requirement of FGF during blastula stages, most likely provided the absence of WNT, and later inhibition of BMP during gastrulation (Linker and Stern, 2004; Delaune et al., 2005; reviewed by Stern, 2005). The issue of the acquisition of definitive neural character remains unresolved, although recent results point to an important role for chromatin remodelling events (Papanayotou et al., 2008).

In the meantime, work has also been done on the intracellular events that take place during neural induction. Upon signalling by the extracellular factors widely described here above, the targeted ectodermal cells need to transduce the signal and interpret it. At least two transcription factors of the POU family have been directly linked to ultimate cell decisions towards acquisition of a neural character: XlPOU2, a noggin-inducible gene (Witta et al., 1995; Matsuo-Takasaki et al., 1999), and XOct-25, inhibitor of ectodermal cell competence to respond to BMP signalling (Takebayashi-

Suzuki et al., 2007). Additionally, a whole cascade of nuclear events leading to *Sox2* activation has been unravelled, involving ERNI (early response to neural induction), a Hensen's node-inducible gene expressed coincidentally with neural induction in the chick and FGF-dependent (Papanayotou et al., 2008). Finally, continued suppression of Smad1 and Smad2 activity, both downstream intracellular effectors of the BMP signalling transduction pathway, has also been shown to be a requirement for neural induction (Chang and Harland, 2007). However, others have been unable to reproduce these results and have proposed alternative interpretations (De Almeida et al., 2008).

Mesoderm induction

In 1969 Pieter Nieuwkoop changed developmental biologists' view on the formation of the middle germ layer of triploblastic animals, called mesoderm. Combining a piece of vegetal pole yolky cells with another piece of animal pole cells from the urodele 2000-celled blastula from which the prospective mesoderm region had explicitly been removed, he demonstrated that mesoderm can form from an interaction between vegetal and animal pole cells (Nieuwkoop, 1969a; reviewed in Gerhart, 1999). As mesoderm did not form from either vegetal or animal pieces alone, but only when both cell types were recombined, he concluded that the mesoderm is a product of an induction event requiring both vegetal and animal cells (Nieuwkoop, 1969a; reviewed in Gerhart, 1999). Furthermore, he and collaborators showed that not only the mesoderm, but also pharyngeal endoderm and part of the gut roof, can originate from animal pole cells, which are transformed by inducers released by the vegetal pole cells (Nieuwkoop, 1969b; Nieuwkoop and

Ubbels, 1972; reviewed in Gerhart, 1999). Nieuwkoop even identified two different vegetal inducing regions: the ¼ most dorsal side of the vegetal pole, which has the ability to induce animal cells to form dorsal mesendoderm (Spemann's organiser), and the remaining ¾ of the vegetal hemisphere, which can induce ventrolateral mesoderm (Boterenbrood and Nieuwkoop, 1973; reviewed in Gerhart, 1999). Remarkably, they found that such induction ends just before the beginning of gastrulation (Boterenbrood and Nieuwkoop, 1973; reviewed in Gerhart, 1999). Later Nieuwkoop and collaborators showed that the same induction event also applies to mesoderm formation in anurans (Sudarwati and Nieuwkoop, 1971; reviewed in Gerhart, 1999) suggesting that mesoderm induction is conserved in amphibians and perhaps chordates (Gerhart, 1999). Altogether, these experiments and the corresponding interpretations meant a conceptual reconsideration on the origin of the mesoderm, at least in vertebrates; for before this time, the middle germ layer had been thought to arise from a pre-established region intrinsic to the structure of the egg, just like the ectoderm and the endoderm; instead, Nieuwkoop indicated that the latter layers and particularly their interaction are the raw ingredients of the mesoderm (Gerhart, 1999).

Some two decades after Nieuwkoop's embryological experiments on induction of the amphibian mesoderm, the molecular nature of the vegetal inducers began to be unveiled. Three main groups of signalling factors and their corresponding transduction pathways have been shown to be at work in the formation of mesoderm.

An FGF (bFGF) was the first substance shown to be capable of eliciting mesoderm induction on *Xenopus* laevis animal caps, mimicking action of the vegetal signal (Slack et al., 1987). Because heparin could block mesoderm formation in whole embryos, a heparin-binding growth factor like bFGF was proposed as the endogenous inducer (Slack et al., 1987). Subsequently, maternal supplies of both the mRNA coding for a *Xenopus laevis bFGF* homologue and its corresponding product were detected in the oocyte (Kimelman and Kirschner, 1987; Kimelman et al., 1988). Overexpression of a dominant negative *FGF* receptor in the frog embryo further demonstrated a requirement of this signalling pathway for mesoderm formation (Amaya et al., 1991). In fact, maternal supplies of *bFGF* in combination with a maternal ubiquitous presence of *FGF* receptors throughout early development, suggests regulation of the pathway at post-transcriptional level (Musci et al., 1990). This may be mediated by changes in the relative levels of functional to non-functional receptor splice variants (Gillespie et al., 1995; Paterno et al., 2000). Finally, FGF was shown to be required for expression of the *Xenopus laevis Brachyury* homologue (Schulte-Merker and Smith, 1995), a T-box gene implicated in mesoderm formation in the mouse, which is found throughout the *Xenopus laevis* marginal zone in all prospective mesodermal cells and is a direct target of mesoderm induction (Smith et al., 1991).

In parallel to the identification of bFGF as an endogenous mesoderm inducer, members of the TGF-β group of signalling molecules were also shown to have similar properties in regard to mesoderm formation. *Vg1* was first identified, as a member of the TGF-β family expressed in the vegetal hemisphere of the *Xenopus laevis* oocyte (Weeks and Melton, 1987);

together with the simultaneous finding that TGF-β activation leads to mesoderm induction in animal pole cells (Kimelman and Kirschner, 1987), this pointed to *Vg1* as a good candidate to collaborate with FGFs in formation of the mesoderm. *Vg1* mRNAs were detected as maternal factors localised in the vegetal cortex and subject to translational control through their 3' UTR (Wilhelm et al., 2000; Colegrove-Otero et al., 2005) and their products were found to be only functional as a mature protein, requiring cleavage of its N-terminal domain in the endoplasmic reticulum (ER) for its release and mesoderm-inducing activity on animal cap cells (Thomsen and Melton, 1993; Dale et al., 1993; Kloc and Etkin, 1994; Deshler et al, 1997). Experimental evidence in the chick showed that in the amniote embryo *Vg1* is not only expressed predicting and colocalising with the forming primitive streak, but its ectopic expression initiates formation of secondary axes (Seleiro et al., 1996; Shah et al., 1997). In the zebrafish, maternal transcripts of the *Vg1* orthologue (zDVR-1) are expressed throughout the embryo (Helde and Grunwald, 1993); however, only overexpression of mature protein leads to ectopic induction of dorsal mesoderm (Dohrmann et al., 1996). In the mouse, expression of *Vg1* orthologues starts prior to gastrulation, and null-mutants for GDF1 (Wall et al., 2000), GDF3 (Chen et al., 2006) or double null-mutants for GDF3 and GDF1 (Andersson et al., 2007), display phenotypes with important mesoderm malformations. Despite all this evidence, the requirement of maternal *Vg1* for mesoderm formation *in vivo* has only recently been demonstrated by functional knockdown with specific antisense RNAs as well as a morpholino in *Xenopus laevis* embryos

(Birsoy et al., 2006). In particular, *Vg1* appears to be involved in the establishment of the dorsal (organiser) mesoderm (Birsoy et al., 2006).

But *Vg1* turned out not to be the only member of the TGF-β family with mesoderm induction capabilities. The fact that overexpression of a truncated form of an *activin* receptor in *Xenopus laevis* leads to inhibition of mesoderm induction (Hemmati-Brivanlou and Melton, 1992), together with localisation of Activin protein in early blastula embryos of different vertebrate species (Mitrani et al., 1990; Albano et al., 1993), including *Xenopus laevis* (Thomsen et al., 1990), led to the idea that *activin* itself may also be involved in this process (Hemmati-Brivanlou and Melton, 1992). However, transfilter experiments with soluble follistatin protein (Slack, 1991) and overexpression of the mRNA for this specific Activin inhibitor (Schulte-Merker et al., 1994), combined with evidence that a truncated activin receptor (Hemmati-Brivanlou and Melton, 1992) also inhibits induction by *Vg1*, suggested that the mesoderm formation properties assigned to *activin* could be explained by its sharing receptors with *Vg1* (Schulte-Merker et al., 1994) or other TGFβs.

*Nodal*, another member of the TGF-β family, was identified in the mouse as a gene expressed around the node during gastrulation (Zhou et al., 1993). In *Xenopus laevis*, 6 homologous genes have been identified: *Xnr1*, *Xnr2* and *Xnr4* are expressed overall in the vegetal hemisphere and progressively accumulate in the dorsal region (Jones et al., 1995), whereas *Xnr5* and *Xnr6* are exclusively found in the dorsal vegetal region (Takahashi et al., 2000) and *Xnr3* is restricted to the superficial layer of the organiser (Smith et al., 1995). All of these, with the exception of the organiser restricted neural

inducer *Xnr3* (Smith et al., 1995; Hansen et al., 1997), possess mesoderm induction capabilities (Jones et al., 1995; Lustig et al., 1996; Joseph and Melton, 1997; Takahashi et al., 2000). Furthermore, ectopic expression of a dominant negative cleavage version of *Xnr2* inhibits mesendoderm formation, a phenotype that can be rescued by co-injection of intact *Xnr2* mRNA (Osada and Wright, 1999). Overexpressing different doses of the C-terminal fragment of *Cerberus*, which provides a specific block of nodal signalling (Agius et al., 2000), suggests a requirement of a gradually increasing higher levels of nodal proteins for formation of progressively more dorsal-anterior mesendoderm (Agius et al., 2000). Analysis of the zebrafish *squint* and *cyclops* mutants, which map to two different nodal homologues, indicates a conserved role for nodal signalling in mesendoderm formation (Feldman et al., 1998). Both allelles are expressed in the extraembryonic yolk-syncytial layer (YSL) as well as in the embryonic marginal blastomeres in the dorsal side of the embryo (Feldman et al., 1998, Chen et al., 2000). Interestingly, localised RNA degradation in the YSL shows that both domains of expression are dependent on extraembryonic signals in the form of mRNA, and that such depletion affects only formation of the ventrolateral mesendoderm, whereas shield (organiser mesoderm) formation depends rather on β-catenin stabilisation (Chen et al., 2000). Finally, mutation of the mouse *nodal* gene confirms a requirement for this signalling molecule for mesendoderm induction (Pfendler et al., 2001).

Wnt signalling, and particularly mobilisation of β-catenin downstream of the canonical pathway, is the third main determinant of mesoderm induction. Overexpression of *int-1* (*Wnt1*) in *Xenopus laevis* was first shown to induce

axis duplications (McMahon and Moon, 1989). Later on, underexpression of β-catenin was seen to inhibit dorsal mesoderm formation (Heasman et al., 1994). Localisation of β-catenin in the dorsal side of the early embryo seems to act in cooperation with TGF-β signalling to establish the so-called Nieuwkoop centre - a transient region in the dorsal vegetal blastula that induces the organiser in the overlying cells - by activating expression of the homeobox gene *Siamois* (Lemaire et al., 1995; Carnac et al., 1996; Crease et al., 1998). A homologous mechanism has been proposed for chick, where *Wnt8* is required together with *Vg1* for primitive streak formation (Skromme and Stern, 2001). Furthermore, evidence has been provided for a requirement of dorsally located β-catenin in formation of the Spemann's organiser in *Xenopus laevis* (Nishita et al., 2000) and zebrafish (Maegawa et al., 2006). On the other hand, the unexpected finding that artificial depletion of a pool of maternal β-catenin localised throughout the marginal zone of the *Xenopus laevis* blastula blocks induction of ventrolateral as well as dorsal mesoderm (including expression of *Brachyury*) upstream of FGF and nodal, suggests that requirement of the Wnt/β-catenin pathway is not restricted to the organiser mesoderm (Schohl and Fagotto, 2003).

Integration and coordination of these different signals is crucial for establishment of the three germ layers and for a proper body pattern to arise. This is especially complex when it comes to signalling by the TGF-β members, because different ligands seem to be involved and there is considerable overlap in their binding affinity to the different receptors (Frisch and Wright, 1998) present in the early embryo. However, detailed characterisation of the transduction pathways to each of these signals and of

the interactions between them has allowed a certain degree of understanding on how mesoderm is formed.

A current model proposes a source of Nodal signalling emanating from the vegetal hemisphere (Osada and Wright, 1999) (extraembryonic YSL in the case of zebrafish) and acting as a morphogen to induce mesoderm at a distal end (Chen and Schier, 2001). Binding of Nodal proteins to ActR-I (activin-like receptor I) transmembrane receptors requires the presence of EGF-CFC type coreceptors; ActRI heterodimerises with ActRIIB (Activin-like receptor II), and the activated complex triggers intracellular transduction of the corresponding pathway (Cheng et al., 2003 and references therein). Long-range inhibition by Lefty, another member of the TGF-β family, which is essential for proper mesendoderm formation (Chen and Schier, 2002; Feldman et al., 2002), may modulate the propagation and/or intensity of the Nodal signal. FGF acts either in parallel or downstream of Nodal signals to promote and restrict mesoderm formation in cells that would otherwise develop into endoderm upon exposure to high levels of Nodal (LaBonne and Whitman, 1994; Cornell and Kimelman, 1994; Roadaway et al., 1999; Mathieu et al, 2004; Maegawa et al., 2006). In *Xenopus laevis*, evidence from both expression patterns and loss-of-funcion analysis suggests that *FGF* acts by rendering cells in the blastula marginal zone competent to mesoderm-inducing signals (Cornell et al., 1995). A mature form of Vg1 acts together with Wnt/β-catenin to promote formation of the Nieuwkoop centre, which in turn will induce the Spemann organiser (Carnac et al., 1996; Crease et al., 1998). Likewise, a combination of *Wnt8* and *Vg1* is sufficient and required to induce axial structures (hence organiser) in the chick

epiblast (Skromme and Stern, 2001). Vg1, like Nodal (and Nodal homologues), signals via ActR-I and/or ActR-II and requires EGF-CFC coreceptors (Cheng et al., 2003), although the roles of these two TGF-β members in mesoderm formation don't fully overlap. Whereas *nodal* seems to be involved in induction of the overall mesendoderm, *Vg1* is a strong inducer of axial structures (Thomsen and Melton, 1993) and its depletion affects mainly formation of notochord and head structures (Birsoy et al., 2006).

In *Xenopus laevis*, an additional TGF-β member called *Derrière* has been identified that localises to the ventral side of the vegetal hemisphere and is required for posterior and ventral mesoderm formation (Sun et al., 1999). Additionally, *Xenopus laevis VegT*, a T-box gene with maternal mRNAs ubiquitously present in the cortex of the vegetal hemisphere is absolutely required for mesoderm induction (Lustig et al., 1996; Zhang and King, 1996; Stennard, 1996; Horb and Thomsen, 1997). Maternal *VegT* is essential for expression of *nodal* homologues, other *TGF-β* members and *FGF* (and *Brachyury*) (Clements et al., 1999; Kofron et al., 1999).

By the late blastula, the region of prospective mesoderm has been delimited and these cells possess distinctive characteristics of the middle germ layer, a timing that corresponds to the induction period demonstrated by Nieuwkoop and collaborators (Botterenbrood and Nieuwkoop, 1973; reviewed in Gerhart, 1999). This prospective mesoderm, albeit pre-patterned, will acquire different identity traits according to positions along the dorsal-to-ventral axis of the embryo. Patterning of the *Xenopus laevis* marginal zone preceding and coinciding with gastrulation seems to be

achieved by two opposing gradients. In the dorsal marginal zone, the nascent Spemann organiser expresses Noggin (Smith and Harland, 1992), Follistatin (Hemmati-Brivanlou et al., 1994; Hemmati-Brivanlou and Melton, 1994) and Chordin (Sasai et al., 1994) transcripts, three secreted molecules that bind and inactivate BMPs (Zimmerman et al., 1996; Iemura et al., 1998; Piccolo et al., 1996). In the ventrolateral marginal zone, *BMP4* transcripts are abundantly expressed in a domain roughly complementary to the aforementioned organiser mRNAs (Hemmati-Brivanlou and Thomsen, 1995). Antagonism between Noggin, Follistatin and Chordin proteins secreted from the dorsal marginal zone on the one hand, and BMP proteins emanating from the opposite side of the prospective mesoderm on the other, generate gradual levels of BMP availability and activity, increasing from ventral to dorsal positions (Holley et al., 1995). An analogous mechanism has been suggested in the zebrafish (Schmid et al., 2000; Dick et al., 2000; Mintzer et al., 2001). Interpretation of the ensuing gradient by a downstream transduction of the signal with sensitivity for different thresholds can be turned into a morphogenetic readout; this is mediated by transcription factors like the homeobox protein Mix.1 (Mead et al., 1996).

Cells simultaneously exposed to different signals may be able to distinguish between those by receptor affinity, but they also have to elicit a comprehensive ultimate response, which is elaborated at the intracellular level. *Smads* in particular, as the nuclear effectors of different TGF-β signals, play an important part in these decisions. In *Xenopus laevis* up to 8 different *smads* have been identified (reviewed in Blitz and Cho, 2009). Smad1, smad5 and smad8 transduce specifically in the BMP pathway

(Ketzschmar et al., 1997; Kramer et al., 2002), although *smad8* has been suggested to modulate the amplitude and duration of BMP signalling in dorsal-ventral patterning both by activating and dampening its activity (Nakayama et al., 1998a). On the other hand, *smad2*, *smad3* and *smad6* appear to transduce activin-like signals (e.g. Nodal, Derrière, Vg1) (reviewed in Blitz and Cho, 2009), the former 2 acting as activators and the latter as a repressor of the pathway (Nakayama et al., 1998b). *Smad6* may restrict neural induction at the edge of the neural plate (Nakayama et al., 1998b). *Smad7* seems to repress transduction of both TGF-β types of signals (i.e. blocking smad1 and smad2 activity) (Nakao et al., 1997; Chang and Harland, 2007; de Almeida et al., 2008) and may be important in neural as well as mesoderm induction (Chang and Harland, 2007; de Almeida et al., 2008), although it may also regulate activities other than *smad1* and *smad2* (de Almeida et al., 2008). Finally, smad4 is an essential co-factor of all other *smads* and acts by triggering their nuclear localisation (Lagna et al., 1996; Zhang et al., 1997; reviewed in Blitz and Cho, 2009), which provides an important crossing point for integration of activin-like and BMP activities in cells simultaneously exposed. In fact, smad4 has been shown to mediate antagonism between the two types of TGF-β during dorso-ventral patterning of the mesoderm, when ventralising BMP and dorsalising Nodal (nodal homologues) signals coincide in the marginal zone, BMP2/4 activity being transduced by smad1 and nodals in turn by smad2. Smad4 may be a limiting factor, for which smad1 and smad2 have to compete (Candia et al., 1997). Nevertheless, initial distinction between these two TGF-β types of signals may start at the level of receptor specificity. Indeed, despite the reported

overlap of their respective ligands in receptor binding affinity, specific receptors have been identified that exclusively bind to either BMPs (XBMPRII) (Frisch and Wright, 1998) or to activin-like molecules (e.g. nodal homologues) (XALK4) (Chang et al., 1997). Expression of XBMPRII during gastrulation is restricted to the marginal zone with lower levels in the dorsal side, and overexpression of a truncated dominant-negative form induces neuralisation of the ectoderm and dorsalisation of the mesoderm (Frisch and Wright, 1998). On the other hand, expression of XALK4 is equally distributed throughout the marginal zone at gastrula stages and overexpression of a truncated dominant-negative form affects mesoderm but not neural induction Chang et al., 1997).

**A-P patterning**

Retinoids

There is considerable evidence that signalling via active retinoids (vitamin A metabolites) is important for early patterning events during development of the vertebrate central nervous system (CNS) (reviewed in (Durston *et al.*, 1998)). Vertebrate embryos go through a sensitive period, starting during gastrulation, when the developing CNS is drastically posteriorised by exposure to the active retinoid all-trans-retinoic acid (RA) (Durston *et al.*, 1989; Sive *et al.*, 1990; Avantaggiato *et al.*, 1996). This agent mimics the action of an endogenous intercellular signal which patterns the developing CNS at this stage (Doniach, 1995; Lumsden and Krumlauf, 1996; Durston et al., 1998). Key regulatory genes involved in CNS patterning are transactivated directly in neuroectoderm by specific binding of retinoic acid receptor (RAR) and retinoid receptor (RXR)

heterodimers to retinoic acid responsive elements (RAREs) (Marshall *et al.*, 1996). Among the retinoid targets are the *Hox* genes, which are crucial for patterning the posterior CNS (hindbrain and spinal cord). Retinoid-regulated *Hox* genes are situated 3' in *Hox* clusters (Lumsden and Krumlauf, 1996; Durston *et al.*, 1998; Gavalas and Krumlauf, 2000). Members of *Hox* paralogous groups (pg) 1-5 are activated by ectopic RA, while members of pg 6-9 are not (Godsave *et al.*, 1998; Bel-Vialar *et al.*, 2002).

To identify developmental functions of retinoids, one approach is to examine the consequences of blocking retinoid signalling. This has been possible by a variety of approaches in different vertebrates, for example: vitamin A starvation in quails (Maden *et al.*, 1996), blocking RAR/RXR transactivation by ectopic expression of dominant negative RAR receptors in *Xenopus* (Kolm *et al.*, 1997; Blumberg *et al.*, 1997; van der Wees *et al.*, 1998), blocking synthesis of active retinoids by mutation of the mouse gene for the enzyme retinal dehydrogenase 2 (*Raldh2*) (Niederreither *et al.*, 1999; Niederreither *et al.*, 2000) or mutation of the zebrafish *Raldh2* gene (Begemann at al., 2001; Grandel *et al.*, 2002), overexpression of the RA catabolising enzyme CYP26 in *Xenopus* and zebrafish (Hollemann *et al.*, 1998; Kudoh *et al.*, 2002) and applying RAR/RXR synthetic inactive ligands that competitively prevent normal RA binding in chick and zebrafish (Dupe and Lumsden, 2001; Hernandez *et al.*, 2007). All of these studies uncovered a common phenotype caused by loss of retinoid signalling in early vertebrate embryos: lack of segmentation of the posterior hindbrain (rhombomeres (r) 5-8) and transformation of this tissue into more anterior hindbrain. Therefore retinoid signalling is essential for patterning the posterior hindbrain.

Wnt

The Wnt family consists of several homologues in each vertebate species. Its members are secreted glycoproteins that act as ligands, activating receptor-mediated signal transduction pathways (reviewed in Moon *et al.*, 2002, and references therein). In *Xenopus laevis*, many of these genes have been cloned and display a wide range of often non-overlapping expression patterns, differing in timing as well as in localisation; but only few of them are found in the right tissues and moment to be potentially involved in A-P patterning (Wolda and Moon, 1992; McGrew et al., 1992; Ku and Melton, 1993; Cui et al., 1995). *XWnt3a* and *XWnt1* appear both in the neural tissue, the former gene is expressed in the neuroectoderm during neurula stages and the latter has been assigned a role in the mid-hindbrain boundary formation (discussed below) (Wolda et al., 1993). However, expression patterns and presumably also functions of *Wnt* genes in other vertebrates do not always correlate with their orthologues in *Xenopus laevis* (or they remain unidentified in this species). Some of them are indeed expressed in the chick gastrula in relevant tissues (Chapman et al., 2004), although as far as attained here, no direct evidence for the specific activity in A-P patterning of one only member has been described. In the mouse, the *Wnt-5a* mutant exhibits problems with A-P axis caudal elongation, although it is due to general tissue growth and not to patterning (Yamaguchi et al., 1999). Interstingly, *Wnt3-a* mutants reveal a gain of neural tissue at the expense of paraxial mesoderm (from somites 7-9 caudally) and eventually homeotic transformation of vertebrae coinciding with downregultion of *Cdx1* (discussed below) (Yoshikawa et al., 1997; Ikeya and Takada, 2001). This

may correlate with the early effects of interfering with Wnt signalling on migration of anterior streak cells in the chick (Sweetman et al., 2008).

A good deal of experimental evidence on the endogenous factors responsible for patterning the A-P axis has originated from studies involving *Xenopus laevis*. *XWnt8* is likely to have a prominent role in this process. After binding of Xwnt8 to suitable receptors, intracellular signals are transduced by the canonical Wnt pathway (Darken and Wilson, 2001), which acts through a rise in cytosolic and subsequent nuclear levels of β-catenin, influencing the function of Tcf/Lef transcription factors. Misexpression of synthetic *Xwnt8* mRNA on the ventral side of the embryo, before activation of the zygotic genome, leads to formation of a secondary axis (Sokol *et al.*, 1991; Smith and Harland, 1991), while later activation of *Xwnt8* expression leads to posteriorisation of the primary axis (Christian and Moon, 1993). In *Xenopus* embryos, it has been shown that β-catenin induced axis formation is mediated via the transcription factor *XTcf3* (Molenaar *et al.*, 1996). The early and late effects of ectopic *Xwnt8* on axis formation can be mimicked by timed activation of an activated form of *XTcf3* (Darken and Wilson, 2001). In *Xenopus laevis*, *Xwnt8* expression is first detected in late blastula stage embryos. Expression is found in all cells of the marginal zone with the exception of the cells centred on the dorsal midline. This pattern of expression in the marginal zone persists during gastrulation (Christian and Moon, 1993). Ectopic expression of *Xwnt8* posteriorises neuroectoderm (Fredieu *et al.*, 1997; Erter *et al.*, 2001; Kiecker and Niehrs, 2001), a feature also known for *Hox* genes (Charite et al., 1994; Hooiveld et al., 1999; Maconochie et al., 1997; McNulty et al., 2006). Conversely, gain-of-function

for *Xdkk1*, a secreted *Wnt* antagonist (Glinka *et al.*, 1998), downregulates expression of *Hoxd1* in neuroectoderm of *Xenopus* embryos (Kiecker and Niehrs, 2001). In mouse and chick embryos, the expression patterns of the *Xwnt8* orthologues have been considered to be indicative of a possible function in the regulation of expression of labial-type *Hox* genes. In chick embryos, the expression of *Cwnt8C* immediately precedes the localisation of *Hoxb1* expression to rhombomere 4 (Hume and Dodd, 1993). In mouse embryos, expression of *Mwnt8* is found in the presumptive rhombomere 4 region (Bouillet *et al.*, 1996). In *Caenorhabditis elegans*, Wnt/Wg signalling elements are involved in the regulation of *ceh-13*, the labial ortholog in this worm (Streit *et al.*, 2002). Furthermore, expression of *Cwnt8C* in the chick coincides with that of *Hoxb1* at the onset of gastrulation in the nascent primitive streak, the site of ingression of mesodermal and endodermal cells, and remains expressed across this structure during gastrulation (Hume and Dodd, 1993; Skromne and Stern, 2001). These properties make *Xwnt8* a good candidate to fulfil the role of initiator of *Hox* expression in marginal zone mesoderm of *Xenopus laevis* embryos.

FGF

The fibroblast growth factor (*FGF*) family is the other (next to retinoids and Wnts) main group of signalling molecules that has been linked to regional specification of the A-P axis in vertebrate embryos. FGF proteins bind to transmembrane receptors that are able to transduce the signal by activating a variety of intracellular pathways (reviewed in Bryant and Stow, 2006). In *Xenopus laevis* initial evidence based on animal cap assays, where ectodermal “non-neuralised” early gastrula cells are isolated from their

surrounding tissues, showed that exposure to bFGF not only induces neural differentiation but also directly (i.e. without inducing mesoderm) imparts posterior neural character (Kengaku and Okamoto, 1995; Lamb and Harland, 1995). In one of these studies, it was proposed that an FGF gradient from the involuting organiser mesodem could be responsible for providing all A-P identities along the axis, increasing doses transforming progressively to more posterior character, as they found that bFGF induces anterior and posterior markers (*XeNK-2*, *En2*, *XHoxc6* and *XHoxb9*) (Kengaku and Okamoto, 1995). The other study suggested a cooperation between noggin and FGF, because the animal cap ectoderm acquired a posterior neural character (*XHoxb9*) upon exposure to FGFs alone or anterior character (*Otx-2*) by being exposed singly to noggin, but it expressed a whole range of molecular markers encompassing the length of the A-P axis when noggin was applied in combination with FGFs (*Otx-2*, *En2*, *Krox-20* & *XHoxb9*) (Lamb and Harland, 1995).

Furthermore, overexpression of a dominant-negative FGF receptor indicated a requirement of FGF signalling for normal expression of posterior *Hox* genes (Pownall et al., 1996; Pownall et al., 1998), and that this FGF activity is mediated by *Cdx3* (Isaacs et al., 1998).

Differentiation of the A-P axis, particularly in neural tissue, appears to be more complex than initially thought. The transformation step postulated by Nieuwkoop (see Neural induction section in this Introduction) seems to be a tightly modulated interaction between at least the three main signals described here (retinoids, FGFs and Wnt).

Randall Moon's group was the first to demonstrate a requirement for Wnt signalling for A-P patterning of the neural tissue. Overexpression of a dominant-negative *Wnt* construct causes loss of posterior structures and a concomittant anteriorisation of the embryo (McGrew et al., 1997). Using the animal cap assay, they showed that activation of the Wnt pathway (*Wnt3a*) caused posteriorisation of the embryo and the corresponding loss of expression of anterior markers, accompanied by a gain of posterior character (McGrew et al., 1997). Interestingly, repression of anterior neural identity by Wnt was seen to be mediated by FGF (McGrew et al., 1997). They also reported that posteriosation of animal caps by FGF requires Wnt activity (McGrew et al., 1997).

In parallel to the finding that Wnt signallling, like FGFs, is required for caudalisation, a retinoid depletion study by overexpression of a dominant negative RARα demonstrated that RA is essential for acquisition of hindbrain identity, whereas other regions of the axis may require FGF activity (Kolm et al., 1997). Similar conclusions were drawn after investigating the role of the paraxial mesoderm in patterning the overlying hindbrain during neurulation, where grafting experiments demonstrated that *Hoxb4* early expression in rhombomeres 7-8 depends on RA, but another signal emanating from the paraxial mesoderm and still unidentified (PMC) is also indispensable (Gould et al., 1998). In fact, a current view proposes complementary roles for RA and FGFs in patterning the neural A-P axis (Kudoh et al., 2002; Bel-Vialar et al., 2002; Shiotsugu et al., 2004; Shimizu et al., 2006). Regarding the *Hox* genes in particular, a subdivision has been established between p.g. 1-5 and p.g. 6-9 members according to their anterior and posterior expression along

the A-P axis, which seems to respond to RA and FGF signals, respectively (Bel-Vialar et al., 2002). Moreover, restriction of these two *Hox* complementary domains seems to be mediated at least partly by *Cdx* genes (Bel-Vialar et al., 2002; Shimizu et al., 2006). FGF and RA seem to clash and modulate each others activities often in the embryo (Shiotsugu et al., 2004), although both signals are also known to synergise in some situations (Kudoh et al., 2002; Shiotsugu et al., 2004, Nordström et al., 2006).

Work in the chick embryo proposes a more integrative model, where the role of Wnt signalling is incorporated next to FGFs and retinoids. By culturing epiblast explants originating from different positions along the neural plate and dissected at different stages of gastrula and neurula embryos, they provide evidence to support a requirement for both FGF and Wnt signalling in patterning the neural tube all along the A-P axis. The former would have a permissive (non-dose dependent) role, whereas the latter would behave like a real morphogen to impart A-P specific identities, with increasingly more posterior positions in the neural plate being instructed by higher or longer Wnt exposure (Nordström et al., 2002). They suggest that Wnt may correspond to the unidentified paraxial mesoderm signal (PMC) (Gould et al., 1998; Muhr et al., 1999; Nordström et al., 2002). Moreover, a retinoid dependence for expression of genes in the posterior hindbrain and anterior spinal cord was also suggested by these experiments (Nordström et al., 2006). On the other hand, results from *in vivo* experiments suggest that opposing gradients of FGF and retinoids regulate each other and posterior identity in the elongating axis of the chick and mouse embryos (Diez del Corral et al., 2003).

Significantly, the latter chick work and other recent studies conducted in zebrafish and *Xenopus laevis*, point to the paraxial/non-organiser rather than the axial/organiser mesoderm as the source of the morphogens involved in A-P patterning of the neural tissue (Koshida et al., 1998; Storey et al., 1998; Muhr et al., 1999; Nordström et al., 2002; Diez del Corral et al., 2003; Wacker et al., 2004).

Isthmic and hindbrain organising centres

Apart from posterior axial regions, two additional organising centres have been identified in the vertebrate embryo. The so-called isthmic organiser at the boundary between midbrain and hindbrain (MHB) is a conspicuous source of Wnt (Wnt1) and FGF (FGF8) signals. Expression of these genes demarcates the posterior limit of the midbrain and the most anterior border of the hindbrain, respectively (Liu et al., 1999). FGF8 is essential for midbrain formation and polarisation in the mouse (Crossley et al., 1996; Lee et al., 1997). FGF8 can also transform midbrain into anterior hindbrain in mice transgenic for an FGF8 driven by a Wnt1 promoter (Liu et al., 2001). Moreover, in *Xenopus laevis* MHB grafts can induce ectopic isthmic organiser markers in the anterior neural plate (forebrain) (Riou et al., 1998). Conversely, caudal forebrain as well as midbrain explants are respecified to the identity and polarity of their new position when grafted into the vicinity of the MHB (Hidalgo-Sánchez et al., 1999; Liu et al., 1999). However, evidence from different species indicates that FGF8 and Wnt1 may be involved in continuous maintenance of the MHB rather than induction (Riou et al., 1998; Reifers et al., 1998; Shamim et al., 1999; Canning et al., 2007) and ectopic FGF cannot trigger formation of a new isthmus by itself (Riou et al., 1998).

The other recently discovered organising centre is located more posteriorly in the hindbrain, where a discrete domain of FGF8 and FGF3 expression appears in the prospective rhombomere (r) 4 region of the late gastrula (Walshe et al., 2002; Maves et al., 2002). This specific region of the hindbrain seems to be specified at the molecular and the morphological level ahead of the rest of the hindbrain and its FGF signalling properties are essential for identity of at least r 5 and 6 (e.g. *Kreisler/valentino*) (Walshe et al., 2002). FGF3 and FGF8 together with the RA-induced vHnf1 appears to regulate specification of the latter rhombomeres, where local expression of *Kreisler/valentino* transcription shall in turn trigger posterior restriction of r4 identity (Hernández et al., 2004; Aragón et al., 2005).

**Chapter 2**

# General materials and methods

## Handling of *Xenopus laevis* adults and embryos

The evening before obtainment of embryos, an adult female and an adult male were removed from their aquaria and injected with a subcutaneous dose of 0.1 ml and 0.01 ml (a total of 500 and 50 IU) of hCG (human chorionic gonadotropin), respectively. When *in vitro* fertilisation was performed, the injected adult male was sacrificed the morning thereafter by cutting the heart open, only after the animal had been immersed into a beaker containing sedative, and subsequently dissected. The testes were then extracted and a fragment was immediately torn off and spread over a batch of freshly laid eggs, obtained after stripping the adult female. The remaining tissue from the testes was stored in the fridge, up to a week, for use on a new batch of freshly laid eggs (the same female could be stripped a few times per day). These fertilised eggs were then put into an incubator at 14°C and left there to turn for at least 30 minutes. Embryos were then dejellied in 2% L-Cysteine pH~7.5 made in distilled water, for about 10-15 minutes at 14°C; they were rinsed a few times in tap water and subsequently placed into a Petri dish with 0.1×MMR (Marc's modified Ringer solution), until treatment or microinjection.

When *in vivo* fertilisation was performed, the adults were injected as described here above; the same evening they were placed together in a middle size container half-filled with water from the aquaria. The morning thereafter, fertilised eggs were collected and dejellied and handled as

described here above; this operation could be repeated throughout the day, as long as new eggs were being laid and the two adults stayed coupled. From there on, embryos were handled as described here above. At the end of the day, the adult individuals were placed back into their respective aquaria.

**Microinjection, culture and treatment of *Xenopus laevis* embryos**

Glass needles were fabricated by pulling glass borosilicate capillaries (GC100F-10, 1.0 mm O.D. × 0.58 mm I.D, with inner filament, Clark Electromedical Instruments) with a needle puller. For microinjection, embryos were selected and placed with a plastic Pasteur pipette into a dish, provided with a grid, filled with 2% Ficoll in 1×MMR; a glass needle was snapped with forceps and then loaded with a microloader pipette tip (Eppendorf). A microinjector (IM-200, Narishige USA Inc.) was used to calibrate and introduce the desired volume into the embryo. After injection, embryos were kept in the same medium (between 30 minutes and 2 hours), to be eventually replaced into 0.1×MMR, with or without Gentamycin (50µg/ml), and cultured until treated or harvested. Embryos were always cultured at a temperature of 14-21°C.

Synthetic capped mRNA was made using the Ambion mMessage mMachine Kit.

### *Xenopus laevis* embryos manipulation: animal caps, Keller explants and grafts

Prior to manipulation embryos were handled, treated or microinjected (if necessary), and cultured as described here above. Tools for the operations included: sharp and blunt forceps, hair knives (made by pulling off own eyebrow or beard hairs, selected, and fixed to a syringe by inserting the hair bottom into the hollow needle and soaking it with nail polish, then air dried), a glass dish, a glass Pasteur pipette, a plastic Pasteur pipette and small plastic Petri dishes coated with a solution of 10% BSA made in water (soaked for at least 30 minutes and then shortly rinsed).

Embryo operations were performed in a glass dish filled with 1×MMR. Vitelline membranes were removed by holding the embryo with blunt forceps and piercing the membrane with a pair of sharp forceps; it was then torn apart by pulling, taking care not to damage the embryo proper.

Animal caps were excised with a sharp hair knife, by holding a stage 9-9½ embryo (vitelline membrane previously removed) with blunt forceps; animal pole facing up, a large square piece was cut from the middle of the pigmented region and then trimmed, to make sure a healthy explant containing only prospective ectodermal cells was taken. For animal cap to animal cap recombinants, two pertinent caps were excised, as here above; the operations were performed immediately after one another; both explants were then transferred with a glass Pasteur pipette to a coated Petri dish filled with 1×MMR, where they were wrapped together and allowed to heal. After 30-60 minutes half of the medium was replaced with 0.1×MMR, and about 20 minutes later most of the liquid was taken off and the dish refilled

with 0.1×MMR (Important note: a lot of care should be taken prevent the explants from getting in contact with the air). The recombinants were then cultured overnight in an incubator at 14°C, to be harvested the next morning. Keller explants were basically made as previously reported (Keller and Danilchik, 1988), with but few variations. Organiser mesoderm was obtained from stage 10 embryos (vitelline membrane previously removed). The embryo was turned with blunt forceps so that the vegetal pole stayed facing up and then slightly tilted to have best accessibility on the dorsal blastopore lip (Spemann organiser). The thin epithelium covering the non-involuted mesoderm was peeled off from the organiser region, with the aid of a sharp hair knife. Two not very deep cuts were made perpendicular to the blastopore lip (still with the same tools), starting at either end of it and going all the way to the animal cap. The embryo was then turned onto its marginal zone, leaving the dorsal side face up, so that a cut uniting the two perpendicular ones could be made (parallel to and opposite the dorsal lip). Finally, the sides and the top of the organiser mesoderm being detached, the tissue flap was bent down by pulling the loose ends outwards (as of a castle wooden gate), while carefully separating it from the deep lying endoderm; a sharp cut was made along the dorsal blastopore lip and the freed organiser tissue was trimmed on all sides to ensure no carryover of other tissues. The explant was then transferred with a glass Pasteur pipette to a coated Petri dish filled with 1×MMR. Non-organiser mesoderm explants were obtained following a very similar procedure, except that the embryos used were older (stage 11); these were oriented to have either lateral side of the blastopore lip (non-organiser mesoderm), rather than the dorsal side

(organiser mesoderm), facing up; and the explants tended to be wider than high, because the marginal zone (i.e. the distance between the animal cap and the yolky vegetal cells) is expected to shrink as gastrulation proceeds and the mesoderm involutes. These non-organiser mesoderm explants were further treated just like their organiser counterparts. After some time (usually 30-60 minutes) the mesodermal explants should have rounded up to form a compact piece. A pair of animal caps from the pertinent embryos was then excised, as described here above. Immediately, the different sorts of explants were assembled together in a coated Petri dish: an organiser and a non-organiser mesoderm were placed next to one another onto the inner (non-pigmented) side of an animal cap; the combination was covered with the other animal cap, its inner side facing down; the final combination was wrapped and allowed to heal. Another pair of animal caps was then excised from the pertinent embryos, and the assemblage repeated as long as mesodermal explants were available. After 30-60 minutes in 1×MMR the medium was gradually replaced to 0.1×MMR as described above for animal cap recombinants. The Keller explants were then cultured overnight in an incubator at 14°C, to be harvested the next morning.

Grafts were performed by explanting a piece of non-organiser mesoderm from a donor embryo at approximately stage 11 and implanting it into a pertinent host embryo of approximately the same stage. The explanted pieces of mesoderm were allowed to round up for about 30 minutes. In the meantime, a host embryo was selected, transferred to a coated Petri dish filled with 1×MMR, the vitelline membrane was removed as described here above, and a hole was made somewhere about the lateral side of the

blastopore lip (area where the explant approximately originated from in the donor embryo) by poking and removing tissue with a blunt hair knife and when necessary even forceps. The rounded mesodermal explant was then inserted and pushed into the freshly scraped hole, as to replace the removed tissue. The grafting was repeated on host embryos freshly perforated, as long as explants were available. These grafted embryos were then allowed to heal for 30-60 minutes in 1×MMR and the medium gradually replaced to 0.1×MMR as described here above. Finally, they were incubated at 14°C overnight, to be harvested the next day.

**Whole-mount in situ hybridisation of *Xenopus laevis* embryos**

This protocol was derived from the one previously described by Harland (Harland, 1991); a few minor modifications were however introduced. (See appendix A for detailed protocol description).

**Templates for synthesis of probes**

*Xenopus* antisense DIG-labelled transcripts were prepared from the following templates:

a 1312 bp *Hoxa-1* fragment (*Hoxa-1*); a 666 bp *Hoxb-1* fragment (*Hoxb-1*); *xHoxlab1;* (*Hoxd-1*) (Sive and Cheng, 1991); EST dac02e11 (*Hoxd*-3); a 708 bp fragment containing the complete *Hoxb-4* ORF (*Hoxb-4*); EST XL094L20 (*Hoxd-4*); EST XL045g13 (*Hoxa*-5); a 998 bp *Hoxc-6* fragment in pGEM1 containing a part of the homeodomain and extending into the 3' UTR (*Hoxc-6*); a 470 bp *Hoxb-9* fragment in pGEM3 (*Hoxb-9*); a 1400 bp *Krox-20* fragment (*Krox-20*) (Bradley *et al.*, 1993); a 1500bp *Engrailed-2* cDNA (*En-*

*2*) (Hemmati-Brivanlou *et al.*, 1991); the *xlPOU 2* ORF (*Xlpou 2*) (Witta *et al.*, 1995); the *Xcad-3* ORF (*Xcad-3*) (Pownall *et al.*, 1996; the xCRABP ORF (*xCRABP*) (Dekker *et al.*, 1994); a 220 bp *OTX-2* fragment *(xOTX-2*) (Pannese *et al.*, 1995); pSP73Xbra (*Xbra*) (Smith *et al.*, 1991); and the *Xwnt8* ORF in CS2+.

Chick antisense DIG-labelled transcripts were used for w-ISH on quail embryos; the probes were prepared from the following templates: a 2 Kb *Hoxb1* cDNA and a *Hoxb4* (Bel-Vialar *et al.*, 2002).

**Embedding and sectioning of *Xenopus laevis* embryos and explants in paraffin**

Embryos were collected and fixed in MEMPFA for at least 4 hours at room temperature or overnight at 4°C. They were then washed once for 15 minutes in methanol and stored in the same liquid at -20°C until required. For histology sections, embryos were briefly transferred to 100% ethanol, subsequently cleared in Histo-Clear (National diagnostics) for about 25 minutes at room temperature, followed by graded immersion in paraffin at 60°C and incubation in 100% paraffin overnight at 60°C. Next day samples were embedded and allowed to solidify at 4°C for approximately 1 hour. Prior to sectioning, they were placed outside the fridge in order to accommodate to room temperature. Histological sections were performed at 8μm of thickness, placed on slides pre-coated with BioBond according to the manufacturer (Electron Microscopy Sciences), on top of a drop of distilled water, and then mildly heated on a heating plate until sections were nicely stretched; finally, they were placed into an oven at 37°C, to dry completely.

Sections were deparaffinised with Histo-Clear, gradually immersed into increasing concentrations of ethanol (i.e.: 66% Histo-Clear / 33% ethanol; 66% ethanol / 33% Histo-Clear; absolute ethanol) and subsequently hydrated in decreasing ethanol dilutions (i.e.: absolute ethanol; 66% ethanol in water; 33% ethanol in water; 33% ethanol in TBS; TBS), a couple of minutes in each solution. Before imaging (after immunostaining, in situ hybridisation and/or lineage tracing with TRITC or FITC), sections were mounted in gelvatol with or without DAPCO (fluorescence anti-fading agent). Analysis and photography was performed by means of an AxioPlan 2 Imaging compound microscope and the corresponding software (Zeiss), provided with a TRITC as well as a FITC band-pass filters. Note: for in situ hybridisation on sections, non Super Frost slides were used; Super Frost slides, in spite of the BioBond pre-coating, invariably led to massive detachment of sections during the SSC washes of the second day of in situ hybridisation.

**Chapter 3**

# Retinoid signalling is required for information transfer from mesoderm to neuroectoderm during gastrulation

## Introduction

A conserved set of retinoid-related genes has been implicated directly in laying down the basic organisation of the vertebrate hindbrain. Among these are genes coding for vitamin A metabolic enzymes (Hernández et al., 2007), as well as a network of retinoid-sensitive transcription factors that appear to set up the boundaries between, and the identities of, different rhombomeres (Vesque et al., 1996; Helmbacher et al., 1998; Theil et al., 1998). *Hox* genes and their collinear expression appear to have a prominent role in hindbrain regionalisation. They (at least the most 3' genes of each cluster) are expressed very early in development, in the dorsal neuroectoderm of the gastrula, preceding other genes that have been implicated in hindbrain patterning. During this early activation, *Hox* transcripts are detectable not only in the neuroectoderm but also, and even earlier, in the mesoderm. Surprisingly, little attention has been given to this initial phase of *Hox* expression and much less to *Hox* gene expression in the early gastrula non-organiser (presomitic) mesoderm. Most work to date has concentrated on the later role of *Hox* genes in patterning the hindbrain, while the mesoderm itself has only been studied at later stages, as a source of signals that pattern the overlying hindbrain: heterotopic grafts in avian embryos demonstrated that morphogens emanating from the somites (including RA)

are needed to set up the correct pattern in the adjacent rhombomeres (Itasaki et al., 1996; Gould et al., 1998). Nevertheless, these experiments were performed long after gastrulation, during somite stages and it has not been investigated whether the early activation of *Hox* genes or retinoid signalling from the early gastrula non-organiser mesoderm play a role in hindbrain patterning.

On the other hand, it is also important to reveal the inductive events that lead to appropriate *Hox* expression in the early mesoderm. Indeed, misexpression of some *Hox* genes brings about homeotic transformations in mesodermal derivatives, such as changes in vertebral identity (Ramirez-Solis et al., 1993; Rijli et al., 1995; Subramanian et al., 1995). Interestingly, it was recently shown by means of mutations in regulatory regions of both *Hoxa-10* and *Hoxa-11* that these genes specify the identity of the corresponding vertebrae by their expression in the presomitic mesoderm, rather than in the somites that derive from it (Carapuço et al., 2005), emphasising the importance of the earliest phase of *Hox* expression in the mesoderm.

We use the synthetic retinoid AGN193109 (AGN) to block retinoid signalling early during development of *Xenopus laevis* embryos. This molecule has been characterised as being a high affinity competitive antagonist for all three RAR receptor subtypes (Agarwal *et al.*, 1996). We use in situ hybridisation to compare expression patterns of *Hox* genes (alongside with other A-P axis molecular markers) in embryos incubated with or without AGN. Applying the blocking agent from blastula stages onwards and analysing the embryos at different developmental stages, shows the earliest requirement of retinoid

signalling for the appropriate expression of each gene. Moreover, starting AGN incubation at later stages of development allows us to narrow down the period of *Hox* sensitivity to retinoids. We also analysed Vitamin A-deficient (VAD) quail embryos (deprived of maternal supply of vitamin A and therefore unable to synthesise retinoids) to confirm the results obtained using the AGN approach. Finally, we performed targeted injections of *xCYP26* and *xCRABP* (coding for a RA-degrading enzyme and a cytoplasmic RA binding protein, repectively) to localise the source of retinoid synthesis in the early embryo directly.

## Materials and methods

Retinoic acid and AGN193109 treatments

Treatments with RA (all-trans-retinoic acid) and AGN (AGN193109) were performed by culturing embryos in 1%MMR or 0.1% MBS containing either or both ligands. Stock solutions were prepared by dissolving RA (Sigma) and AGN193109 (Allergan) in DMSO to concentrations of 10-2 M and 10-3 M, respectively, and then stored at -80°C. Final dilutions for embryo incubations were made in 1% MMR or 0.1%MBS. Medium containing the ligands was added at about stage 8 (before gastrulation) or at stage 13 (post-gastrulation treatment) and embryos cultured in these media until harvested.

Luciferase essay

To measure luciferase activity 5-10 embryos were homogenised in 100:1 reporter

lysis buffer (Promega) and mixed with 300:1 assay buffer (0.1 M potassium phosphate buffer pH 7.8 (KPi), 1 mM DTT, 3 mM ATP and 15 mM MgSO4). The luciferase reaction was started by addition of 100:1 0.1 M KPi, 1 mM DTT and 0.4 mM luciferin. Light units were measured during 10 seconds in a luminometer (Biocounter, Lumac).

Immunofluorescence and confocal microscopy

For immunostaining of the CNS, embryos were fixed overnight at 4°C in methanol.

Pigmentation was bleached in 80% methanol, 6% H2O2, 15 mM NaOH, for approximately 1 hour. After bleaching, the embryos were washed four times 15 minutes in PBS containing 0.2% Tween-20, and then blocked for 30 min with PBT (0.2% Tween-20 and 3% bovine serum albumin in PBS). To reveal neural tissue, embryos were incubated overnight in antibodies 2G9 (Jones and Woodland, 1989) and Xen-1 (Ruiz I Altaba, 1992) at 1:1, 2G9 and 1:5 Xen-1 in PBT at 4°C. The embryos were washed four times 30 minutes at room temperature in PBT and incubated overnight in secondary antibody conjugated to the Cy-5 far-red fluorophore (Jackson Research Labs, Inc.) at 4°C. After washing four times 30 minutes in PBS containing 0.2% Tween-20 at room temperature, the embryos were fixed in methanol and cleared in 1:2 benzyl alcohol:benzyl benzoate. The Cy-5 signal was analysed with confocal laser scanning microscopy (CSLM). Approximately 25 optical sections were recorded for each embryo, and reconstructed into one image.

DNA templates and lineage tracers for microinjection

The full length of *xCYP26* in pBSRN3 (K. de Roos), a full-length *xCRABP* in pGEM3 (E. J. Dekker and Tjadine) and a DR5-TATA-luciferase (K. de Roos).

Sulforhodamine dextran (MW 10,000) (S-359, Molecular Probes) was injected in all four macromeres of 8-cell-stage embryos, 1 nl of 5 ng/nl per injection.

Whole-mount in situ hybridisation of chick and quail embryos

In situ hybridisation followed a standard protocol (Stern, 1998).

Quail (wild-type and VAD) embryos were bred and collected by Dr. Maija Zile and collaborators as previously described (Dersch and Zile, 1993; Zile et al., 2000). Embryos were fixed in 4% formaldehyde in PBS and then transferred to absolute methanol, to be shipped by courier on ice. For collection and storage of chick embryos, the procedure described below was followed.

Embryos were collected in calcium-magnesium-free PBS (CMF) and fixed in freshly made 4% formaldehyde/CMF/EGTA for 1 hour at room temperature or overnight at 4°C. Embryos were transferred to absolute methanol and stored in this for up to 1 week at -20$^{o}$C. When embryos had to be kept longer before in situ, they were processed to the end of day one of the in situ hybridisation protocol (until just before adding the probe) and then kept at -20$^{o}$C until needed. When required, the embryos were placed into a water bath at the hybridisation temperature for a few hours, then probe added as if at the end of day one.

## Results

### The general retinoid antagonist AGN193109 impairs retinoid signalling (in collaboration; K. de Roos)

To assess the effect of the RAR antagonist AGN on retinoid signalling in early *Xenopus*, embryos were injected with a DR-5-TATA-luciferase reporter and the embryos treated with $10^{-6}$ M AGN or $10^{-6}$ M RA during gastrulation. Luciferase activity after antagonist treatment decreased to 30% of that measured in untreated or carrier (0.1% DMSO) treated embryos, whereas it increased nearly 4-fold after treatment with $10^{-6}$ M RA (see Figure 3.1). These results show that AGN impairs retinoid signalling *in vivo*.

We investigated if AGN can inhibit teratogenicity of exogenously applied RA. As previously described, treatment of gastrula stage embryos with $10^{-6}$ M RA causes severe anterior truncations, lost or reduced eyes and a reduced cement gland (Durston *et al.*, 1989; Sive *et al.*, 1990) (Fig. 3.2A). Co-application of $10^{-6}$ M RA with $10^{-6}$ M AGN rescues the development of eye pigment and cement gland, similar to embryos treated with AGN only, whereas co-application of $10^{-7}$ M AGN with $10^{-6}$ M RA rescue only partially (Fig. 3.2A). These results show that AGN can antagonise RA teratogenicity.

To determine whether a decrease in retinoid signalling has an effect on axial development, embryos were treated with $10^{-7}$ M or $10^{-6}$ M AGN alone, starting before gastrulation and until harvesting. These embryos showed shortening of the A-P axis, more severe in the higher dose (Figure 3.2A).

**Retinoid loss of function by the general retinoid antagonist AGN generates mid-axial defects (in collaboration; K. de Roos)**

The luciferase assay above showed that treatment with $10^{-6}$ M AGN not only interferes with the effects of exogenously applied RA, but also causes a decrease in endogenous retinoid signalling. This predicts that AGN treatment should also cause an axial patterning phenotype similar to those previously observed using various RA depletion approaches. We then performed immunohistochemistry with antibodies recognising neural antigens: 2G9 (Jones and Woodland, 1989) and Xen-1 (Ruiz i Altaba, 1992); and further confocal microscopy analysis revealed changes in the morphology of the tadpole (st. 45) CNS caused by AGN treatment. Examination of the brain revealed a compressed prosencephalon and mesencephalon. The anterior hindbrain is slightly enlarged and properly segmented, rhombomeres 4 and 5 are quite normal in size but less distinctly segmented, whereas rhombomeres 6, 7 and 8 are truncated or absent (Fig. 3.2B). These findings confirm that AGN causes posterior hindbrain defects similar to those seen using other methods to deplete retinoid signalling.

**The definitive hindbrain defect caused by AGN treatment is established by the late neurula**

We examined the developmental changes in the patterning of the CNS caused by blocking retinoid signalling. By the end of neurulation (st. 20), we observed the following effects of AGN (Fig. 3.3A-G): the *Hoxb-1* anterior domain, normally restricted to the prospective r4, expands towards the future spinal cord (Fig. 3.3A). In contrast, the *Hoxd-3* expression domain (r5-6) is reduced as compared to control embryos (Fig. 3.3B). *Hoxb-4*

expression becomes undetectable in the presumptive hindbrain and the distance between the *En-2* stripe at the mid-/hindbrain boundary and the anterior *Krox-20* stripe (r3) increases, indicating an enlargement of r1-2 (Fig. 3.3C). In control embryos, *Hoxa-5* is expressed in two stripes in the future anterior spinal cord and posterior hindbrain; both stripes disappear after AGN treatment (Fig. 3.3D). On the other hand, retinoid depletion expands the normal *Hoxc-6* expression domain anteriorly (prospective spinal cord), but its intensity appears unaffected (Fig. 3.3E). Expression of *Otx-2 and Xcad3* (markers of fore- midbrain and posterior spinal cord domains, respectively) shows no obvious increase in the overall length of the hindbrain (Fig. 3.3F). Expression of *XlPOU2* (r2; Fig. 3.3G) and *Krox-20* (r3/r5; Fig. 3.3C and 3E) in their respective r2 and r3 domains shows an enlarged anterior hindbrain; the gap of *XlPOU2* expression between prospective r2 and r4 is also expanded, consistent with the expanded anterior *Krox-20* stripe (r3). Furthermore, the r5 stripe of *Krox-20* becomes thicker in AGN treated embryos. The *XlPOU2* stripe associated with r4 is no longer distinguishable, as it fuses with the spinal cord domain, from which it is separated by a small gap in control embryos (Fig. 3.3G). Co-staining for *Krox-20* and *Hoxc-6* confirms that the posterior hindbrain region is severely shortened after AGN treatment (Fig. 3.3E). The correlation between these expression pattern changes and the malformations observed in the future hindbrain of AGN-treated embryos suggests that the action of retinoid signalling on gross A-P patterning of the hindbrain is complete by st. 20.

**The hindbrain defect caused by AGN is more extensive in the early neurula**

Because there is considerable evidence indicating that retinoids affect axial patterning during gastrulation and the coincidental expression of genes responsible for establishing a retinoid signalling domain (Durston et al., 1989; Hollemann et al., 1998; Durston et al., 1998; Swindell et al., 1999; Begemann et al., 2001), we looked for the possible effects of abrogation of RA signalling during earlier stages of development. We chose to focus on a time at which the most 3' located *Hox* genes (paralogues 1-6) would normally be expressed and therefore could be affected by an impaired RA signal. At the early neurula (st. 13) stage, the most 3' of the *Hox* genes examined (*Hoxd-1*, *Hoxa-1*, *Hoxb*-1, *Hoxd-3* and *Hoxb-4*) are strongly affected by reduced retinoid signalling (Figs. 3H-L). However, *Hoxc-6* is unaffected (Fig. 3.3M). Dissection of embryos confirmed that expression of the most 3' *Hox* genes is severely downregulated in the neuroectoderm upon AGN treatment (data not shown).

To compare the length of the presumptive posterior hindbrain with and without AGN treatment, a combination of probes was used for in situ hybridisation. After AGN treatment, the gap between the *Krox-20* stripes and *Hoxc-6* is much smaller (Fig. 3.3M) and, surprisingly, the *Krox-20* stripe at r5 is also greatly reduced (compare with Fig. 3.3C and 3E). This shows that truncation of the posterior hindbrain domain by retinoid depletion is effective already at the end of gastrulation.

To correlate the late hindbrain phenotype in the tadpole with the molecular truncation observed in the young neurula, we treated embryos beginning

either before or after gastrulation and incubated them to stage 45, when they were fixed. AGN treatment before gastrulation causes the phenotype shown in Figure 3.1B. Treatment after the end of gastrulation (st. 13 onwards) however, does not cause gross morphological malformations (results not shown). Surprisingly, post-gastrulation treatment with AGN still causes an alteration, albeit minor, in the molecular pattern of the posterior hindbrain of tadpole embryos. Specifically, expression in the posterior hindbrain of the most anterior *Hox* genes analysed (*Hoxb1* and *Hoxd3*) is not affected by retinoid depletion after the end of gastrulation, but expression of the most posterior ones (*Hoxb4* and to a lesser extent *Hoxb5*) is still sensitive to AGN treatment after this period (Fig. 3.4). The extension of the posterior hindbrain – indicated by the distance between the posterior expression stripe of *Krox-20* in r5 and the anterior expression boundary of *Hoxc-6* in the spinal cord (Fig. 3.4E) – is not changed by AGN treatment after gastrulation, as compared to non-treated embryos. On the contrary, AGN treatment beginning before gastrulation causes a remarkable shrinkage of the region, as was already seen at earlier stages. These results indicate that retinoid signalling is required mainly before the end of gastrulation for the hindbrain to acquire a proper morphology; however, part of its pattern remains flexible and it is still retinoid dependent.

**3' *Hox* gene expression in the neuroectoderm, but not in the mesoderm, requires retinoid signalling during gastrulation (in collaboration; H. Jansen)**

The above experiments suggest that the phenotype observed after AGN treatment in the hindbrain arises during gastrulation, and that retinoid

mediated patterning of the presumptive hindbrain begins at this time. We therefore undertook a more detailed study of the phenotype generated by AGN during gastrulation by analysing both the initiation and the maintenance of *Hox* gene expression during this developmental period.

*Hoxd-1* appears as a "pioneer" gene, its expression first becoming detectable at stage $10^{+}$ (Wacker *et al.*, 2004). AGN treatment does not affect the initial expression in non-organiser mesoderm (Fig. 3.5A and 5B). However, as gastrulation proceeds in normal embryos, *Hoxd-1* expression becomes localised more dorsally and is then transferred onto the neuroectoderm; this pattern is absent in AGN treated embryos. By the end of gastrulation, the expression pattern of *Hoxd-1* is very strongly reduced and remains exclusively in its mesodermal domain upon retinoid inhibition (Fig. 3.5D and 5E). In contrast, when RA instead of AGN is added to the medium, *Hoxd-1* expression is induced in a much larger domain and earlier than in control embryos; expression is particularly strong in the ectoderm and is maintained throughout gastrulation (Fig. 3.5C and 5F).

We then extended the study to *Hoxa-1* and *Hoxb-4*. Both are first expressed weakly at about stage 11 and are upregulated as gastrulation proceeds (Wacker *et al.*, 2004). We analysed the timing of the consequences of AGN treatment on their expression: whereas their initiation in the mesoderm is not affected, embryos fail to develop normal neuroectodermal expression when incubated in AGN throughout gastrulation (data not shown). Unlike the three genes mentioned above, neither *Hoxc-6* (Fig. 3.5G-I) nor *Hoxb-9* (not shown) expression is affected by either gain- or loss of retinoid function at this stage of development.

**Avian embryos also employ a mechanism other than retinoid signalling to regulate early mesodermal *Hox* expression**

The above experiments show that abrogation of the retinoid pathway with a synthetic inhibitor in *Xenopus* embryos impairs the normal neuroectodermal expression of 3' *Hox* genes in the mid-axial region early during development. However, the earliest appearance of the same 3' *Hox* transcripts in mesodermal tissue is not affected by this treatment. To investigate whether such a difference in regulation between the two germ layers is due to limitations of our experimental approach, we turned to the VAD quail model. We performed in situ hybridisation with two 3' *Hox* genes on both VAD and normal quail gastrula and neurula embryos to determine the effects of a thorough depletion of retinoid signal. Expression of *Hoxb-1* and *Hoxb-4* is initiated during gastrulation first in the primitive streak and later in mesodermal cells; this activation is seen in both control and VAD embryos (Fig. 3.6A-F). In the CNS, expression of these genes normally begins only after the start of neurulation (Fig. 3.6G); in VAD quail embryos however, this expression is downregulated in the anterior neural tube (Fig. 3.6H). Thus, the avian embryo provides further evidence that 3' *Hox* genes are regulated differently in the mesoderm and in neural tissue. Furthermore, there is an asynchrony of *Hox* expression between the two tissues, which is much more pronounced in avian than in *Xenopus* embryos.

**Retinoid dependent transfer of information from mesoderm to neuroectoderm in the gastrula**

The above experiments suggest that retinoid signalling may be involved in the transfer of positional information from mesoderm to neuroectoderm in

the hindbrain region at early stages of development. To test this more directly we first injected mRNA encoding xCYP26, which is involved in the degradation of RA (Hollemann *et al.*, 1998), into *Xenopus* early embryos. *xCYP26* mRNA alone or, more effectively, co-injection of *xCYP26* and *xCRABP* (cellular retinoic acid binding protein) mRNAs into *Xenopus* restored the axial defects caused by RA incubation. We conclude that combined ectopic expression of *xCYP26* and *xCRABP* causes retinoid loss of function. To test the idea that retinoid signalling from the mesoderm is involved in hindbrain patterning, we loaded gastrula mesoderm cells but not neuroectoderm cells by injection of *xCYP26* and *xCRABP* into all four vegetal blastomeres (fated to become mesoderm and endoderm, but not neuroectoderm; see Figure 3.8) in 8-cell-stage embryos. This causes dramatic loss of *Hoxa-1* and *Hoxb-1* expression in the neuroectoderm (Fig. 3.7). Together, these results strongly suggest that a retinoid-dependent signal from mesoderm is required for the correct pattern of neuroectodermal *Hox* expression.

## Discussion

The retinoid antagonist AGN193109 gives a strong phenotype in the *Xenopus* CNS, resembling those previously reported using other highly effective approaches to inhibit retinoid signalling (Maden *et al.*, 1996, Kolm *et al.*, 1997; Blumberg *et al.*, 1997; van der Wees *et al.*, 1998, Hollemann *et al.*, 1998; Niederreither *et al.*, 1999; Niederreither *et al.*, 2000, Begemann at al., 2001; Dupe and Lumsden, 2001; Grandel *et al.*, 2002; Kudoh *et al.*, 2002; Hernandez *et al.*, 2007). This phenotype features disturbances of the

posterior hindbrain and anterior spinal cord, and is detectable in its definitive form in late neurula stage embryos. This “mid-axial” phenotype arises during gastrulation. AGN treatment during the gastrula stage already causes disturbances of the gastrula stage neural expression of all 3’ anterior *Hox* genes examined (*Hoxd-1*, *Hoxa-1*, *Hoxb-1*, *Hoxd-3* and *Hoxb-4*), whereas expression of more 5’ posterior *Hox* genes (*Hoxc-6* and *Hoxb-9*) is not affected. Severe changes in gene expression are seen later in the future posterior hindbrain region at the early neurula stage. This early defect is the converse of that caused by early retinoid application (Conlon and Rossant, 1992; Godsave *et al.*, 1998). The defect is more extensive than that observed at later stages, both in this investigation and in previous retinoid loss of function studies. If this difference is due to a recovery occurring at later stages, this must be independent of retinoid signalling, as retinoid inhibitor treatments from the end of gastrulation to larval stages could not prevent it. Auto- and cross-regulation among *Hox* genes is likely to be involved, reflecting a second *Hox* phase of axial patterning in the CNS. It has been demonstrated in both chicken and mouse that there is indeed a second phase of regulation for *Hox* genes, which starts soon after initiation of somitogenesis and is not dependent on retinoic acid but rather on auto-regulation of and interactions between *Hox* genes (Gould *et al.*, 1998).

In our experiments, virtually no trace of 3’ *Hox* mRNA expression (paralogues 1-5) was detectable in the prospective hindbrain region of retinoid antagonist-treated early neurulae (the region that is to recover part of its pattern in the following stages). One possible explanation is that HOX proteins remain in an area and time where the corresponding mRNAs have

disappeared; these HOX proteins would be insufficient to activate early neural *Hox* genes expression by means of the known cis-acting HOX responsive elements, since early expression requires retinoid activation via RAREs (see above). However, once retinoid sensitivity ends and RA-independent auto-regulatory and cross-regulatory elements take over neural regulation of 3' *Hox* genes, HOX proteins remaining in the region could still be available in sufficient amounts to trigger the new phase of *Hox* induction. Another possible explanation is that factors other than HOX proteins themselves are needed to start the second phase of *Ho*x activation in the hindbrain region, for instance FGF (Godsave and Durston, 1997) or WNT (McGrew *et al.*, 1997). If a second activation phase devoid of HOX protein remnants in the region occurs, the ordered array of *Hox* expression observed after partial restoration of the initial mid-axial defects would be consistent with *Hox* collinearity. Whatever the explanation, it appears that during early development the embryo retains a safety mechanism, employing a second round of ordered *Hox* activation to allow possible environmental deficits of vitamin A to be circumvented.

We followed the dynamics of the consequences of retinoid antagonist treatment on *Hox* expression during gastrulation. This is precisely the period in which the first transcripts are detected in all vertebrates studied (Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1996; Wacker *et al.*, 2004). Moreover, in *Xenopus*, expression is initiated in non-organiser mesoderm, before appearing in the overlying neuroectoderm. We found that the antagonist compromises neuroectodermal *Hox* expression, but has no effect on non-organiser mesodermal expression. To confirm our

observations and at the same time test the universality of this phenomenon in vertebrates, we took advantage of the VAD quail model. These embryos lack endogenous retinoid signalling and therefore offer an alternative approach to antagonist-treatment in *Xenopus*. Analysis by whole-mount in situ hybridisation indicated that early mesodermal expression of two 3' *Hox* genes (*Hoxb-1* and *Hoxb-4*) is not affected in VAD embryos, whereas later neural plate expression is impaired; this phenotype is comparable to that observed in *Xenopus* neurulae. Therefore, our evidence from both *Xenopus* and avian embryos indicates that *Hox* regulation in the early paraxial mesoderm is distinct from that in the prospective hindbrain and independent of retinoid signalling. The nature of this regulation is yet to be elucidated.

We hypothesised that a possible early function for retinoid signalling would be to mediate transfer of A-P information (and thus of *Hox* expression) from mesoderm to neuroectoderm. This idea is supported by many other data, including mesodermal location of the RA generating enzyme RALDH2 in different vertebrates (Swindell *et al.*, 1999; Berggren *et al.*, 1999; Haselbeck *et al.*, 1999; Chen *et al.*, 2001; Begemann *et al.*, 2001) together with neural action of retinoid dependent enhancers (Gould *et al.*, 1998). We tested this idea by knocking-out mesodermal retinoid signalling but not neural retinoid signalling by targeting mesoderm precursor blastomeres with mixed mRNAs for xCYP26 and xCRABP, two proteins which mediate retinoid degradation. This treatment effectively eliminates the early neural expression of two 3' *Hox* genes examined (*Hoxa-1* and *Hoxb-1*), indicating that retinoid signalling is required for a "vertical" signal (corresponding to part of Nieuwkoop's

"transformation" signal) generated by mesoderm and which induces 3' *Hox* genes in neuroectoderm.

The present experiments in *Xenopus* indicate that the retinoid-mediated component of the "transformation" signal acts before the end of gastrulation and arises from non-organiser mesoderm (which includes the prospective non-segmented paraxial mesoderm). However, experiments in avian embryos have suggested that the later somitic mesoderm can also signal to impart pattern onto the hindbrain in a retinoid-dependent way (Itasaki *et al.*, 1997; Gould *et al.*, 1998). Moreover, regionalisation of the avian posterior hindbrain seems to coincide with the beginning of somitogenesis (Nordström *et al.*, 2006). This timing difference could be due to distinctive characteristics of each species: in *Xenopus* the mesoderm migrates as a sheet (involution) which may allow for an early and robust source of signal to pattern the overlying neuroectoderm during gastrulation, whereas the cell-by-cell mode of mesoderm migration in the avian embryo is less favourable for local delivery of the morphogen (RA). We suggest that in *Xenopus* retinoids emanate from the non-segmented non-organiser mesoderm to pattern the overlying neuroectoderm along with gastrulation movements, whereas avian embryos undergo this process later, after re-epithelialisation of the mesoderm into somites can provide a robust retinoid signal to the adjacent neural tissue. This signal might directly mediate a positionally specific vertical instruction or it might have an auxiliary function (Gould *et al.*, 1998). Whatever the mechanism, the mode of action (mesoderm to neuroectoderm) and nature (RA) of the signal appears to be common to both species and it correlates well with the predicted properties of part of the "transformation"

signal that Nieuwkoop proposed a long time ago for amphibians (Nieuwkoop, 1952).

***Figure 3.1.*** **The endogenous retinoid activity in *Xenopus laevis* embryos is reduced by the RAR antagonist AGN193109.** Embryos were injected with a DR-5-luciferase reporter construct and cultured in 1% MMR containing 0.1% DMSO (solvent control), $10^{-6}$ M AGN or $10^{-6}$ M RA (positive control) from stage 9 to 13. Luciferase activity was analysed by measuring 8 pools of 5 stage 13 embryos. Values shown are average values, represented as relative luciferase activity (non-injected control (NIC) is set at 1). Error bars represent the s.e.m. (in collaboration; K. de Roos).

***Figure 3.1***

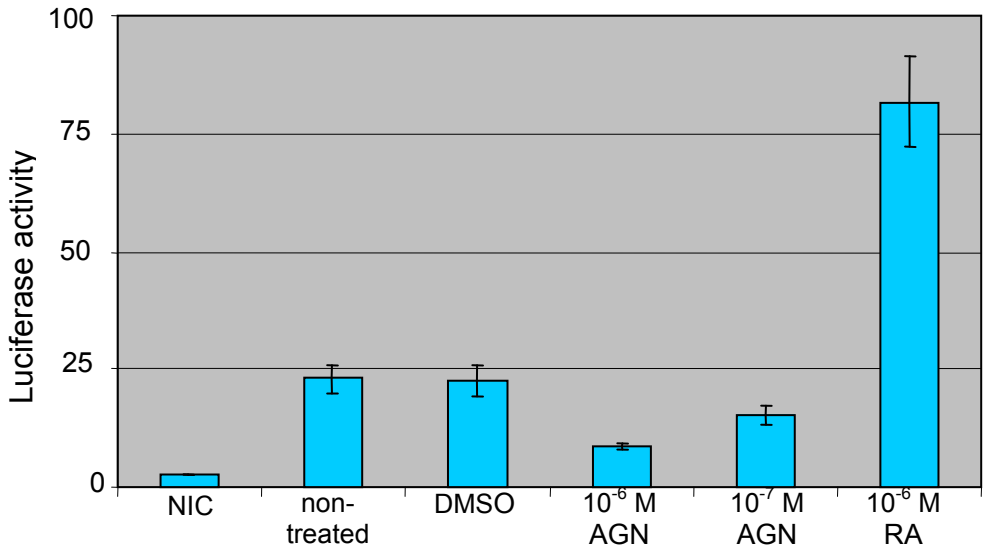
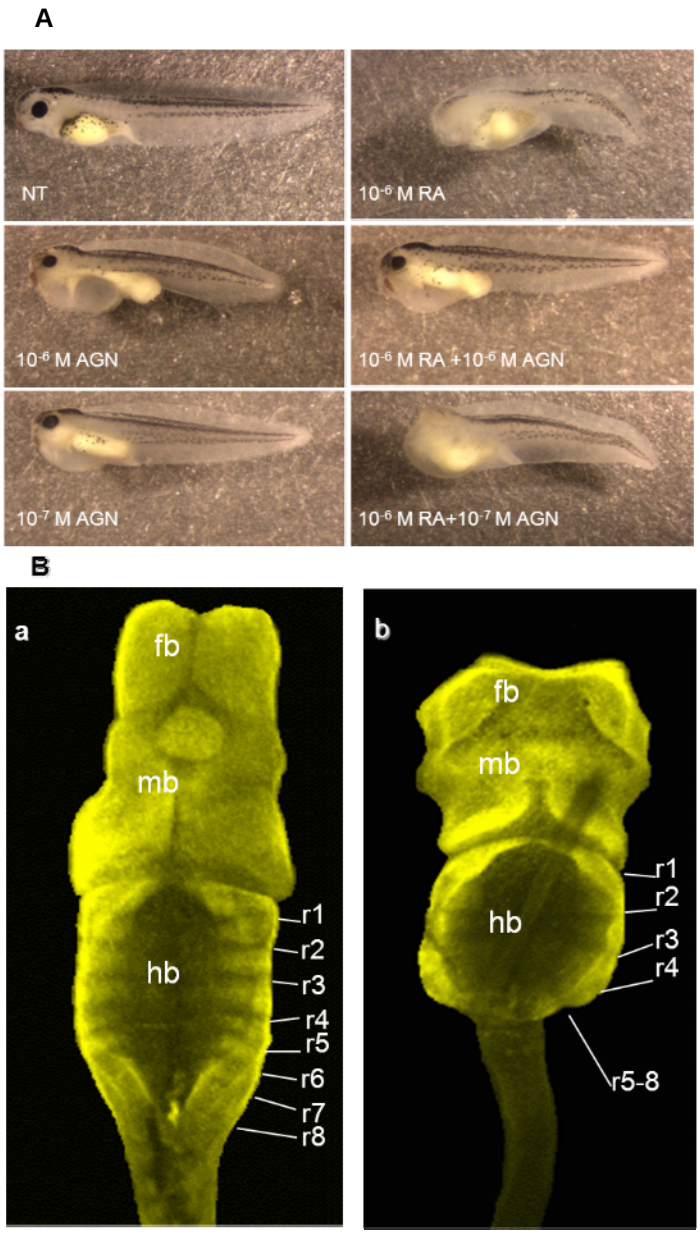

100
75
50
25
0
Luciferase activity
NIC
non-treated
DMSO
10-6 M AGN
10-7 M AGN
10-6 M RA

***Figure 3.2.A*** **The RAR antagonist AGN193109 rescues the RA phenotype.** *Xenopus laevis* embryos incubated in $10^{-6}$ M RA show anterior truncations. Embryos incubated in $10^{-6}$ M and $10^{-7}$ M AGN show a shorter hindbrain area and a large heart oedema with the phenotype being more severe at $10^{-6}$ M. When embryos are incubated in equal concentrations ($10^{-6}$ M) of RA and AGN the resulting phenotype is more like the AGN phenotype. When $10^{-6}$ M RA is combined with $10^{-7}$ M AGN the phenotype is more like an RA phenotype. NT (control). (**B**) AGN treatment caused severe brain malformations. CLSM images of stage 45 tadpole brains labelled with Xen1 and 2G9 antibodies. (a) Control embryo, treated with 0.1% DMSO. (b) $10^{-6}$ M AGN treated embryos (**fb**: forebrain; **mb**: midbrain; **hb**: hindbrain, rn refer to rhombomere numbers). In AGN treated embryos, the number of rhombomeres was reduced to 4 or 5. (in collaboration; K. de Roos).

***Figure 3.2***


A
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10-6 M RA
10-6 M AGN
10-6 M RA +10-6 M AGN
10-7 M AGN
10-6 M RA+10-7 M AGN
B
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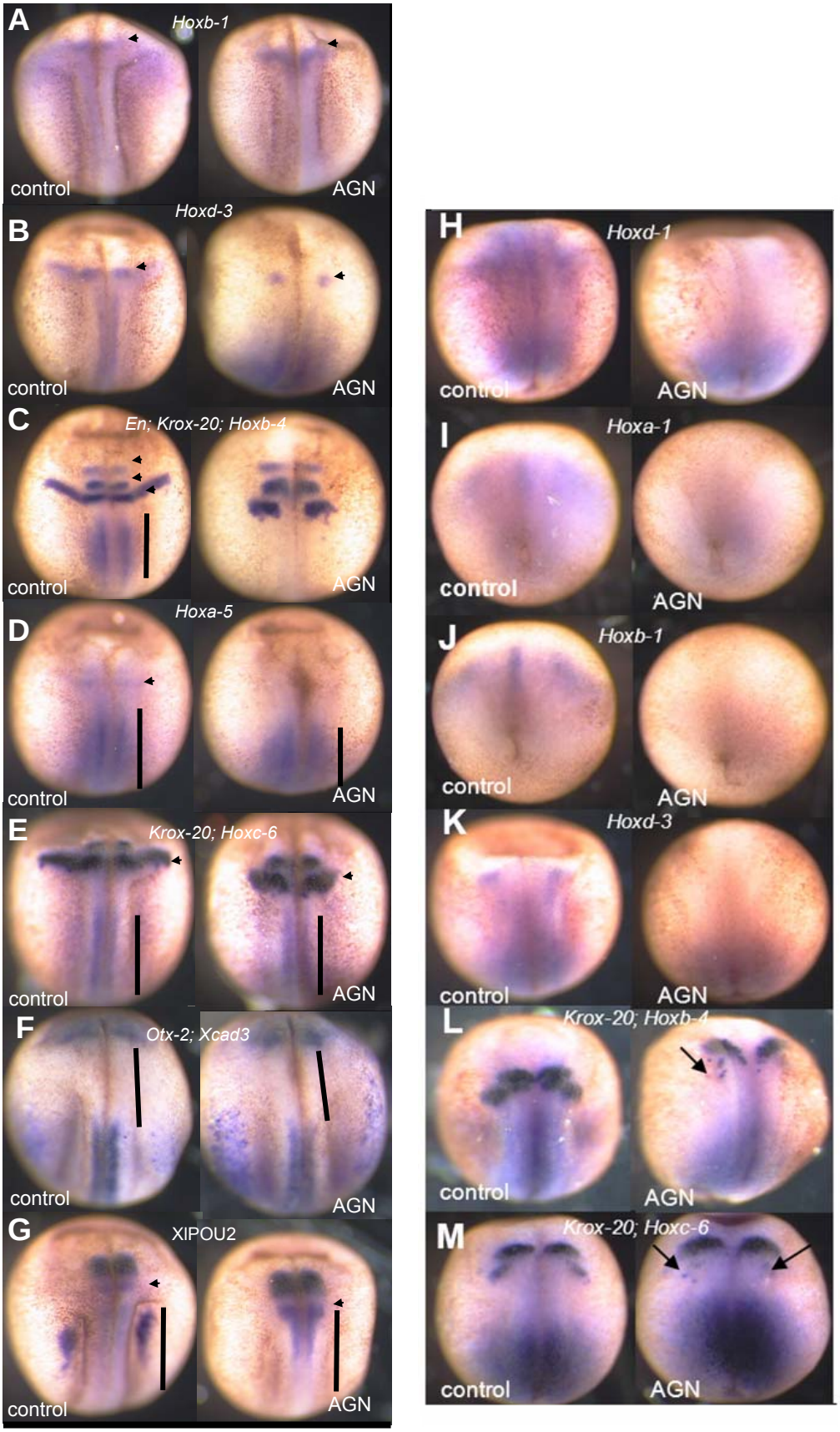
fb
mb
hb
r1
r2
r3
r4
r5
r6
r7
r8
b
fb
mb
hb
r1
r2
r3
r4
r5-8

***Figure 3.3*. Retinoid depletion causes radical molecular truncation of the posterior hindbrain by the end of gastrulation.** Left panel shows whole-mount in situ hybridizations (wISH) on st. 20 *Xenopus laevis* embryos (**A-F**). The left row shows non-treated embryos (indicated by control) and the right row embryos treated with $10^{-6}$ M AGN (indicated by AGN). All views are dorsal and anterior at the top. (**A**) *Hoxb-1*, arrowhead indicates hindbrain expression; (**B**) *Hoxd-3*, arrowhead indicates hindbrain expression; (**C**) *En2*, *Krox-20* and *Hoxb-4*, top arrowhead indicates *En* stripe, bottom arrowheads indicate *Krox-20* stripes and bar indicates *Hoxb-4* stripe; (**D**) *Hoxa-5*, arrowhead indicates hindbrain expression and bar indicates spinal cord expression; (**E**) *Krox-20* and *Hoxc-6*, arrowhead indicates posterior *Krox-20* stripe, bar indicates *Hoxc-6* expression; (**F**) *Otx-2* and *Xcad3*, bar indicates gap between *Otx-2* (anterior) and *Xcad3* (posterior) expression patterns; (**G**) *XlPOU2*, arrowhead indicates hindbrain expression and bar indicates spinal cord expression.

Right panel shows whole-mount in situ hybridizations on st. 13 *Xenopus laevis* embryos (**H-M**). The left row shows non-treated embryos (indicated by control) and the right row embryos embryos treated with $10^{-6}$ M AGN (indicated by AGN). All views are dorsal and anterior at the top. (**H**) *Hoxd-1*; (**I**) *Hoxa-1*; (**J**) *Hoxb-1*; (**K**) *Hoxd-3*; (**L**) *Krox-20* (anterior stripes) and *Hoxb-4*; (**M**) *Krox-20* (anterior stripes) and *Hoxc-6*. Arrows in pictures **L** and **M** localise sparse cells representing the posterior stripe of *Krox-20*.

***Figure 3.3***


A
Hoxb-1
control
AGN
B
Hoxd-3
control
AGN
C
En; Krox-20; Hoxb-4
control
AGN
D
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control
AGN
E
Krox-20; Hoxc-6
control
AGN
F
Otx-2; Xcad3
control
AGN
G
XIPOU2
control
AGN
H
Hoxd-1
control
AGN
I
Hoxa-1
control
AGN
J
Hoxb-1
control
AGN
K
Hoxd-3
control
AGN
L
Krox-20; Hoxb-4
control
AGN
M
Krox-20; Hoxc-6
control
AGN

***Figure 3.4.*** **The molecular identity of the hindbrain is determined by retinoid signalling mostly during gastrulation.** Whole-mount in situ hybridizations on tadpole (st. 32) *Xenopus laevis* embryos. The upper row shows non-treated embryos; the middle row embryos treated with $10^{-6}$ M AGN from the blastula until the point of fixation; the lowest row embryos treated with $10^{-6}$ M AGN from st. 13 until the point of fixation. All views are lateral. (**A**) *Hoxb-1*, (**B**) *Hoxd-3*, (**C**) *En2, Krox-20 and Hoxb-4*, (**D**) *Hoxa-5*, (**E**) *Krox-20* and *Hoxc-6*. Arrows point to the anterior expression border of each *Hox* gene.

***Figure 3.4***

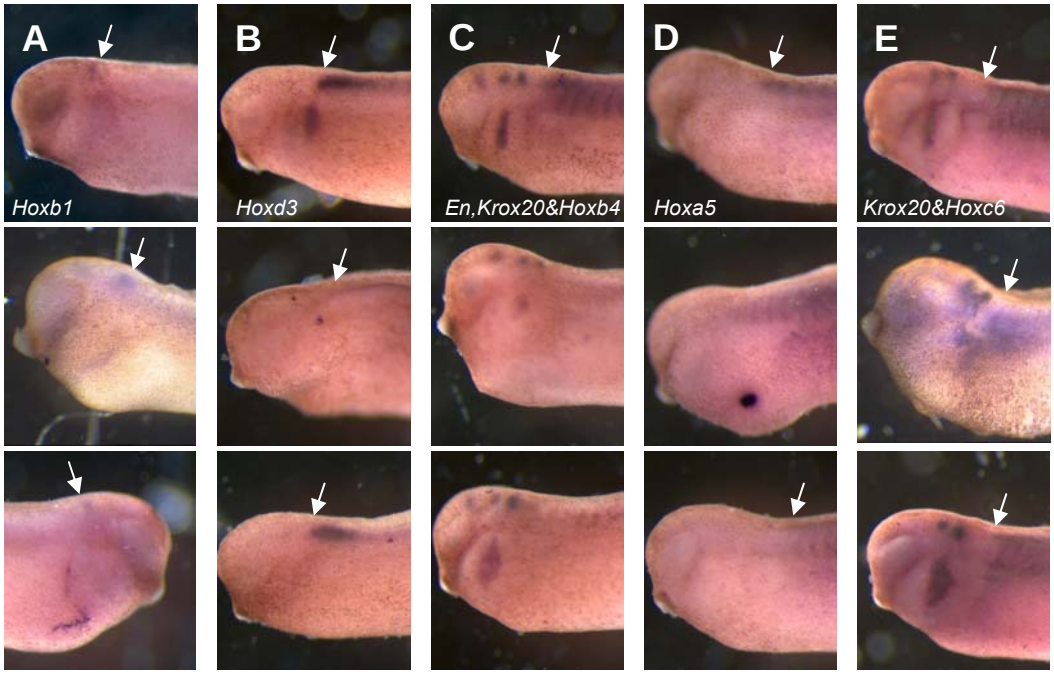


***Figure 3.5*. AGN incubation affects early expression of *Hoxd-1* in the neuroectoderm but not in the underlying mesoderm.** Whole-mount in situ hybridizations on *Xenopus laevis* embryos. *Hoxd-1* expression at st. 11 (**A-C**) or st. 12.5 (**D-F**). Embryos were incubated with $10^{-6}$ M AGN (**A**, **D**), $10^{-6}$ M RA (**C**, **F**) or not treated (0.1% DMSO) (**B**, **E**). After photographing the embryos were cut along the indicated dashed line and a lateral view of the cut surface is shown next to the right of each embryo. Arrows in 3**D** and 3**E** point to the faint mesodermal expression remaining at that stage (mostly non-involuted mesoderm). *Hoxc-6* expression on stage 12 embryos (**G-I**). Treatment with $10^{-6}$ M AGN (**G**), $10^{-6}$ M RA (**I**) or not treated (**H**). (in collaboration; H. Jansen).

***Figure 3.5***

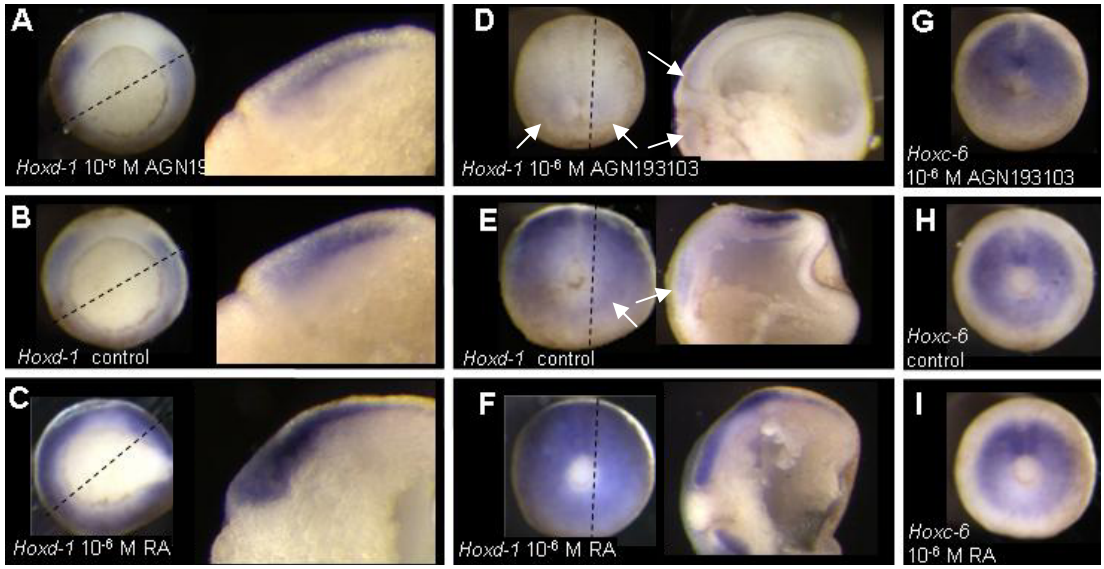

A
Hoxd-1 10-6 M AGN19
D
Hoxd-1 10-6 M AGN193103
G
Hoxc-6
10-6 M AGN193103
B
Hoxd-1 control
E
Hoxd-1 control
H
Hoxc-6
control
C
Hoxd-1 10-6 M RA
F
Hoxd-1 10-6 M RA
I
Hoxc-6
10-6 M RA

***Figure 3.6.*** **Initial mesodermal *versus* later neural plate expression of *Hoxb-1* and *Hoxb-4* in VAD and wild-type quail embryos.** Whole-mount in situ hybridizations on quail embryos. *Hoxb-1* and *Hoxb-4* expression patterns in quail embryos. Wild-type HH st. 4 early (**A**) and late (**C**) are compared to equivalent VAD (**B**) and (**D**) embryos; both show expression of *Hoxb-1* in the primive streak and later in migrating ingressed cells. *Hoxb-4* expression patterns are also shown for HH st. 4 wild-type (**E**) and VAD (**F**) embryos, along the primitive streak. At HH st. 8 *Hoxb-4* expression pattern includes the neural tube in wild-type (**G**) but not in VAD (**H**) embryos. All views are dorsal.

***Figure 3.6***

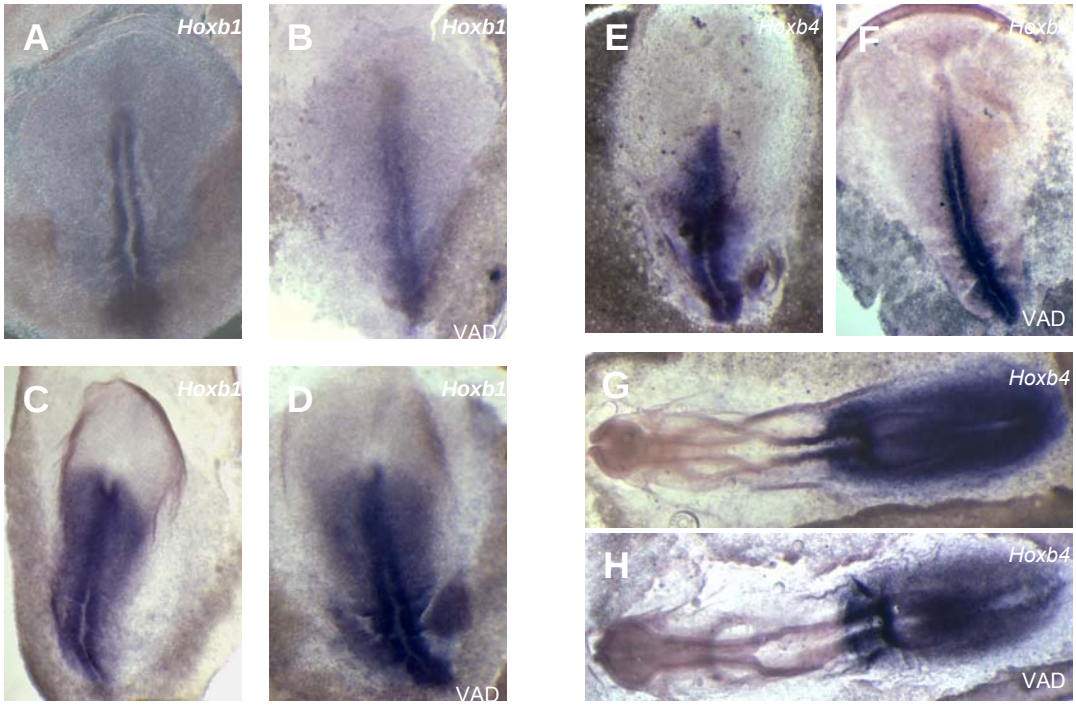

A
Hoxb1
B
Hoxb1
VAD
E
Hoxb4
F
VAD
C
Hoxb1
D
Hoxb1
VAD
G
Hoxb4
H
Hoxb4
VAD

***Figure 3.7*. Effects of targeted retinoid signalling removal in the mesoderm.** Whole-mount in situ hybridizations on *Xenopus laevis* embryos. *Hoxa-1* (**A**) and *Hoxb-1* (**B**) expression at st. 13. NIC: non-injected controls. xCYP26 and xCRABP: Injection of 100 pg x*CYP26* : 100 pg x*CRABP* mRNA four times, one time into each macromere at 8-cells stage. Whole embryos (top picture) are shown in a dorsal view with anterior being up. Cut embryos (bottom picture) are shown in a lateral view.

***Figure 3.7***

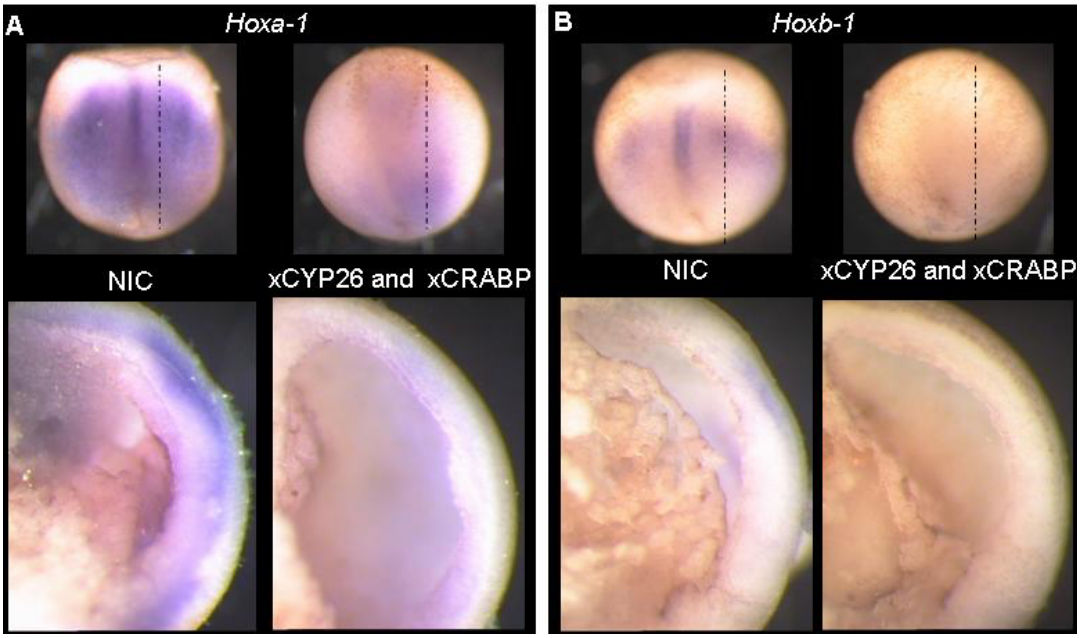
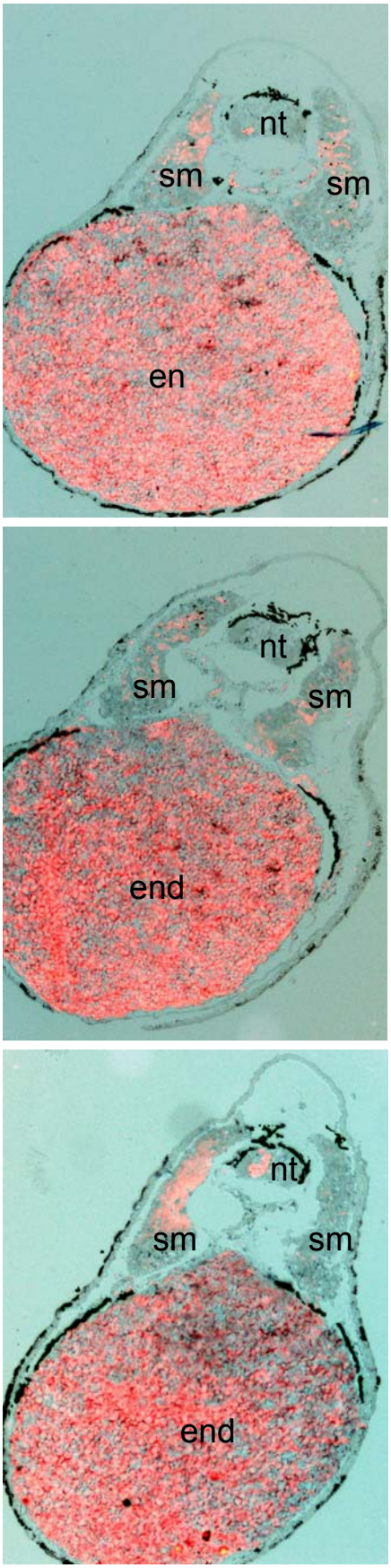


***Figure 3.8.*** **Localisation of rhodamine dextran in the tadpole after targeted injections in the 4 macromeres at 8 cells stage.** Transversal histological sections along the antero-posterior axis of st. 40 *Xenopus laevis* tadpoles. Rhodamine dextran was injected in the 4 macromeres of 8 cells stage embryos. Images show lineage tracing, where the red colour signal corresponding to the fluorescence emitted by rhodamine molecules has been superposed onto a dark interference contrast image of the same section. Sections of three different embryos show the distribution of the rhodamine dextran, which is mostly confined to the endoderm (end) and the somitic mesoderm (sm), but only in some few scattered cells it appears in the neural tube (nt).

***Figure 3.8***



**Chapter 4**

# *Xwnt8* directly initiates expression of labial *Hox* genes

## Introduction

We were interested in the initiation of *Hox* expression initiated in the gastrula embryo. To date, there is no information on what triggers the earliest phase of *Hox* expression, which occurs in the non-organiser mesoderm (see General Introduction). Three signalling pathways have been implicated in conferring regional identity along the A-P axis early during development. Retinoids (Lumsden and Krumlauf, 1996; Durston *et al.*, 1998; Gavalas and Krumlauf, 2000) and FGFs (Godsave and Durston, 1997; Bel-Vialar *et al.*, 2002) have been shown to regulate expression in the neural tissue of anterior and posterior *Hox* genes, respectively. The relevance of WNTs for *Hox* induction and their possible mechanism of action is less well understood (Kiecker and Niehrs, 2001), although it is clear that inhibition of Wnt underlies the most rostral identity in the nervous system (Houart et al., 1998; Houart et al., 2002; Kaspsimali et al., 2004). Because there is little information on whether Wnt signalling, and particularly the Wnt canonical pathway, might play a role in initiating the earliest *Hox* gene expression in the non-organiser mesoderm, we set out to investigate this.

First, we studied in detail the dynamics of the early expression of *Xwnt8* because it is one of the best candidates among the members of the Wnt family to be participating in early A-P patterning (see General Introduction) and examined whether this coincides with the expression of *Hoxd1*, *Hoxb4*,

and/or *Hoxc6* during gastrulation. These three genes were chosen because they are expressed in well-defined spatial domains in the early neuroectoderm, corresponding to mid-hindbrain (the identities of rhombomeres 4 and 5) (*Hoxd1*, Kolm and Sive, 1995b), posterior hindbrain (*Hoxb4*, Harvey and Melton, 1988), and anterior spinal cord (*Hoxc6*, Oliver *et al.*, 1988; De Robertis *et al.*, 1989), respectively. In addition, the spatiotemporally collinear expression of these genes in ventrolateral mesoderm has been described (Wacker *et al.*, 2004). We found a significant overlap in expression between *Xwnt8* and the *Hox* genes examined in ventrolateral mesoderm during gastrula stages.

Next, we analysed the effects of *Xwnt8* loss-of-function, using a morpholino-based strategy, on development and on the expression of several early *Hox* genes during gastrulation. Besides, we performed *Xwnt8* gain-of-function experiments. To investigate whether the observed effects on *Hox* expression by Wnt8 gain-of-function are direct, we used a fusion of an activated form of *XTcf3* to the ligand-binding domain of the glucocorticoid receptor, which allows hormonal regulation of nuclear translocation and thus control of the timing of activation of this pathway. Throughout this study, special attention was paid to the timing and localisation of the effects brought about by manipulation of the Wnt canonical pathway, as they are likely to give specific information on tissue specificity.

## Materials and methods

Cycloheximide and dexamethasone treatments

Treatments with Cycloheximide (CHX) and Dexamethasone (DEX) were performed at a concentration of 10 μM. The final dilution was made up in 0.1×MMR from a 1000×CHX or a 500×DEX frozen stocks. Medium containing CHX was applied around stage 9½, followed by addition or co-addition of DEX 30 minutes later. Embryos were incubated in their pertinent media until harvested, 4 hours after starting the CHX treatment (Kolm and Sive, 1995a).

DNA constructs, DNA templates and morpholinos for microinjection

TVGR (Tcf3 Δaa1-87 (β-catenin binding domain-VP16-GCR-CS2) (Darken and Wilson, 2001) mRNA was injected into the animal pole of embryos as 100pg at the one-cell stage or 50pg per blastomere at the 2-cell stage.

The CS2-*Xwnt8* construct was made by cloning in the CS2+ (Rupp *et al*., 1994) the full-length coding region of *Xwnt8*, obtained by PCR using the CSKA-X8 plasmid (Christian and Moon, 1993) as template and the following primers: f: 5'-gaggaattccggatagcagccttcatcatgcaaaacacc, r: 5'–ctactcgagtctccggtggcctctgttcttcc, (containing an *EcoR*I and an *Xho*I restriction site, respectively), using the restriction sites in the primers. 50 pg, in a volume of 8 nl, of this plasmid was injected, dissolved in water, into the animal pole of embryos at the one-cell stage.

*Xwnt8* morpholino ($MO^{Xwnt8}$) supplied by Gene Tools, LLC, has the sequence: 5'-tttgcatgatgaaggctgctatccg. The $MO^{contr}$ has the sequence: 5'-cctcttacctcagttacaatttata. Embryos were injected into the animal pole at the one-cell stage with 32 or 64 ng $MO^{Xwnt8}$ in a volume of 4 or 8 nl respectively,

or with the $MO^{contr}$ using the same conditions. For rescue experiments an *Xwnt8*-MO-insensitive construct was used (MOI CS2*Xwnt8*) that does not contain the morpholino binding site. There are a total of 7 mismatches between the MO and its (now non-complementary) binding site. Also, while the concentrations of MO used were 32 and 64 ng/ml, those of the construct were 50 pg. Taking into account the respective molecular weights of these two reagents, about a 20000 fold difference in molarity is delivered. Stoichiometric titration of the MO by this construct or the mRNA transcribed from it is inconceivable.

Morpholino stocks were prepared by dissolving the powder in Gurdon's buffer to a concentration of 1mM, directly aliquoted and then stored at -20°C. Immediately before use, an aliquot was thawed, diluted in water, then vortexed and heated at 65°C for 5 minutes, and finally centrifuged to prevent blockage of the needle by morpholino salts during microinjection.

RNA extraction, RT-PCR and primers

Total RNA was extracted using Tri-Pure reagent (Roche). First strand cDNA was subsequently synthesised using Superscript KSII polymerase (Gibco-BRL) and primed with Oligo dT15 according to the manufacturer's instruction. RT-PCR assays were performed in the exponential phase of amplification as described (Busse and Séquin, 1993) using Tfl polymerase (Promega) in buffer containing 20 mM TrisAc, pH 9.0, 75 mM KAc, 10 mM $NH_4SO_4$, 1.7 mM $MgSO_4$ and 0.05% Tween-20.

The primers used were: *Hoxd1*: f: 5'-agggaactttgcccaactctcc r: 5'-gtgcagtacatgggtgtctggc; *Hoxa-1*: f: 5'-atgtggacctgtccctagcagc r: 5'-tgctttgcagctcaatgagacc; *Hoxb-1*: f: 5'-tttggttgtcttgggaggatttct r: 5'-

ataatggggatggaaggtttgttg; *Hoxb4* (Hooiveld *et al.*, 1999); *Hoxb-5*: f: 5'-cgtcagtctcggaggagg r: 5'-aatgtgagcggctcatacag; *Hoxc-6* f: 5'-cagagccagacgtggactattcatccagg r: 5'-caaggtaactgtcacagtatggagatgatggc; *Hoxc-8* f: 5'-cacatgttacaacgccgaggccacc r: 5'-gagtgtgagttccttgctctccttagtctcctcttcctc; ODC f: 5'-gtcaatgatgggtgtatggatc r: 5'-tccattccgctctcctgagcac.

## Results

### *Xwnt8* and anterior *Hox* genes have partially overlapping expression domains during gastrulation (in collaboration; P. in der Rieden)

If *Xwnt8* is involved in the initiation of *Hox* gene expression, it needs to be co-expressed with *Hox* genes. Because Wnt family members are secreted factors their functional domains could extend beyond the borders of their mRNA expression domains, but overlapping or neighbouring expression of *Xwnt8* and *Hox* genes could reveal functional relations. We compared in detail the expression patterns of *Xwnt8* and three early *Hox* genes in gastrula and early neurula stage embryos.

Early during gastrulation *Xwnt8* is expressed in a horseshoe-like pattern in the mesoderm, with a gap corresponding to the organiser mesoderm (Fig. 4.1A). As gastrulation progresses, *Xwnt8* expression expands in the animal direction (Fig. 4.1B and 1C). Expression of *Xwnt8* is lost at the ventralmost side of the embryo around stage 12 (Fig. 4.1C) but is maintained in dorsolateral mesodermal domains close to the blastopore, and in involuted mesoderm (Fig. 4.1C). During early neurulation, three domains of *Xwnt8* expression can be observed on either side of the midline: in the paraxial

mesoderm, in the presumptive hindbrain neuroectoderm, where *Xwnt8* expression anterior boundary coincides with the anterior expression domain in paraxial mesoderm, and a posterior domain in dorsolateral mesoderm (Fig. 4.1E).

Expression of *Hoxd1* starts in a horseshoe-like pattern in the marginal zone mesoderm at stage 10.25 (Fig. 4.2B) and two dorsolateral domains become prominent as gastrulation progresses (Fig. 4.2B). At stage 11.5, the ectoderm overlying the dorsolateral mesodermal expression domains starts to express *Hoxd1* (Fig. 4.2B). Early during neurulation (St.12.5), expression of *Hoxd1* can be found anteriorly in ectoderm, and in lateral mesoderm extending backwards to the almost closed blastopore (Fig. 4.2B). The expression patterns of *Hoxd1* and *Xwnt8* in gastrula stages clearly overlap (compare Fig. 4.2A to 2B). During early gastrulation, the overlap can be found in marginal zone mesoderm. At stage 13, both genes are expressed in the neuroectoderm. In paraxial and ventrolateral mesoderm, expression of *Xwnt8* is within the domain of *Hoxd1* expression (compare Fig. 4.2A to 2B).

Initiation of *Hoxb4* expression during gastrulation takes place later than *Hoxd1* initial expression (stage 10.5), but in a similar nested domain in marginal zone mesoderm (Fig. 4.2C). At stage 12, the ectoderm overlying the dorsolateral mesodermal expression domains starts to express *Hoxb4* (Fig. 4.2C). At stage 13, this ectodermal expression is located more posteriorly than the ectodermal expression of *Hoxd1* (compare Fig. 4.2B to 2C). Expression of *Hoxb4* overlaps with that of *Xwnt8* in marginal zone mesoderm (compare Fig. 4.2A to 2C). At stage 12 this overlap is restricted to the dorsolateral domain of *Hoxb4* expression; at stage 13 *Xwnt8* and

*Hoxb4* are co-expressed in paraxial mesoderm, while no overlap can be observed in neuroectoderm. Expression of *Hoxc6* is initiated in a similar pattern to that of *Hoxd1* and *Hoxb4*, starting at stage 11.5 in marginal zone mesoderm (Fig. 4.2D). At stage 13 expression of *Hoxc6* can be observed in ectoderm overlying the dorsolateral mesodermal expression domain (Fig. 4.2D), with its anterior expression boundary located posterior to the most anterior expression of *Hoxb4* (compare Fig. 4.2C to 2D). The expression patterns of *Hoxc6* and *Xwnt8* overlap in marginal zone mesoderm but not in neuroectoderm. The overlap in posterior dorsolateral mesoderm persists during later gastrula stages (compare Fig. 4.2D to 2A).

These results are consistent with the possibility that *Xwnt8* could serve a role as an initiator of *Hox* gene expression in the non-organiser mesoderm during gastrulation.

### *Xwnt8* loss-of-function leads to anteriorisation of embryos and loss of *Hoxa1*, *Hoxb1* and *Hoxd1* expression as well as of other *Hox* genes (in collaboration; P. in der Rieden)

To investigate whether *Xwnt8* is of importance for the early expression of *Hoxd1*, *Hoxb4* and *Hoxc6*, as well as for other *Hox* genes, we used a *Xwnt8* morpholino antisense oligonucleotide ($MO^{Xwnt8}$). A number of loss-of-function strategies have been used to study the function of *Xwnt8*: *dnWnt8* (Hoppler *et al.*, 1996), *Xdkk-1* (Glinka *et al.*, 1998), and *Sizzled* (Salic *et al.*, 1997). The advantage of a morpholino-based approach is the high specificity for the ligand as compared to overexpressing antimorphic forms of *Xwnt8* or Wnt antagonists (reviewed in Heasman, 2002).

MO$^{Xwnt8}$ was injected into the animal hemisphere of embryos at the one-cell stage or (in both cells) at the two-cell stage, to deliver the MO$^{Xwnt8}$ all over the embryo; subsequently, the embryos were allowed to develop until control embryos reached stage 24 (Fig. 4.3A) or stage 35 (Fig. 4.3B). Knocking down *Xwnt8* function by injection of MO$^{Xwnt8}$ leads to anteriorisation of the embryo in a concentration dependent manner (Fig. 4.3). In MO$^{Xwnt8}$ injected embryos, the axis is reduced and the cement gland enlarged (compare Fig. 4.3A to 3C and 3D, and 4.3B to 3E). This coincides with the effects reported in previous studies using *Wnt8* loss-of function or depletion of Wnt signal: *dnWnt8* (Hoppler *et al.*, 1996), *Xdkk-1* (Glinka *et al.*, 1998), *Sizzled* (Salic *et al.*, 1997). In zebrafish embryos, injection of morpholinos directed against both the *Zwnt8* ORFs found (Erter *et al.*, 2001; Lekven *et al.*, 2001) leads to comparable effects to those we observed for *Xenopus* using the MO$^{Xwnt8}$. A control morpholino (MO$^{contr}$), unrelated in sequence to MO$^{Xwnt8}$, was injected in the same amounts; this did not cause abnormalities. The specificity of the MO$^{Xwnt8}$ was further shown by rescue of the *Xwnt8* loss-of-function phenotype with CS2-*Xwnt8* (morpholino insensitive, MOI, plasmid DNA; see materials and methods for details). 64 ng of MO$^{Xwnt8}$ and 20 pg of MOI-CS2-*Xwnt8* were injected either singly or in combination into the animal hemisphere of embryos at the one-cell stage. Embryos receiving both the MO$^{Xwnt8}$ and MOI-CS2-*Xwnt8* show a reduction in size of the cement gland as compared to injection of the MO$^{Xwnt8}$ alone (Fig. 4.3F).

After confirming that the MO$^{Xwnt8}$ is a valid *Xwnt8* loss-of-function reagent, we investigated its effects on the expression patterns of 6 *Hox* genes: *Hoxd1*, *Hoxa1*, *Hoxb1*, *Hoxb4*, *Hoxd4* and *Hoxc6*. Mesodermal expression

of *Hoxd1* was strongly downregulated, and the distance between the two dorsolateral domains of expression in marginal zone mesoderm was increased by *Xwnt8* loss-of-function (Fig. 4.4A). Ectodermal expression of *Hoxd1* was also downregulated in injected embryos (Fig. 4.4A). *Hoxa1* was regulated weakly in the early gastrula mesoderm, but apparently not in the early neurula (stage 13) neuroectoderm (Fig. 4.4B). *Hoxb1* is also downregulated in the neuroectoderm at early neurula (Fig. 4.4C). Expression of *Hoxb4* in mesoderm and ectoderm was also modestly altered by *Xwnt8* loss-of-function (Fig. 4.4D). *Hoxd4* was downregulated too (Fig. 4.4E). Expression of *Hoxc6* was ectopically upregulated in dorsal mesoderm of stage 10.5 embryos and in mesoderm and dorsal ectoderm of embryos at stage 12 (Fig. 4.4F-G). *In situ* hybridisation was performed on embryos injected with 64 ng of $MO^{contr.}$. For all markers studied, injection of the control morpholino results in unaltered expression (data not shown). To confirm these results and at the same time extend our analysis, we used RT-PCR to examine the effect of *Xwnt8* loss-of-function on expression of several *Hox* genes (Fig. 4.6). This confirmed the effects on *Hoxa1*, *Hoxb1*, *Hoxd1* and late *Hoxc6* (whereas st. 11 expression levels seemed rather downregulated), but showed a stronger downregulation of *Hoxb4* (at st. 13) and an earlier downregulation of *Hoxb1* (stage 11) at a stage where the *Hoxb1* in situ hybridisation yielded too weak a signal to estimate possible effects of *Xwnt8* loss-of-function. In addition, early *Hoxa7* (at st. 11) and neurula *Hoxc8* and more slightly *Hoxb5* (st.13) were found to be downregulated, *Hoxd4* could not be detected by RT-PCR.

We also determined the effects of the *Xwnt8* loss-of-function on the expression of the organiser mesoderm and anterior neural plate marker *Otx2*. Its expression domain was expanded at stage 10.5 (Fig. 4.4H). These results provide a molecular confirmation of the morphological phenotype observed after injection of *Xwnt8*MO; namely, an anteriorisation of the mid-axial region of the embryo.

**Ectopic expression of *Xwnt8* after the mid-blastula transition leads to an upregulation of expression of *Hox* genes (in collaboration; P. in der Rieden)**

To study the effects of *Xwnt8* gain-of-function on gastrulation and neurulation we designed a construct driving expression of *Xwnt8* after the mid-blastula transition (MBT). This avoids the early, dorsalising activity found following *Xwnt8* synthetic mRNA injections (Smith and Harland, 1991; Sokol *et al.*, 1991). To this end, we generated a plasmid containing the full-length coding region of *Xwnt8* in the CS2+ vector (Rupp *et al.*, 1994) and named the construct CS2-*Xwnt8*. The CS2+ vector harbours a sCMV promoter leading to efficient expression and subsequent translation of the derived mRNA in *Xenopus* embryos after the MBT (Turner and Weintraub, 1994; Kühl *et al.*, 1996). Embryos at the one-cell stage were injected into the animal hemisphere with 100 pg of CS2-*Xwnt8* plasmid; this results in strong posteriorisation (Fig. 4.3F). Next, we assayed for the early expression of the same *Hox* genes examined above, *Otx2*, the mesodermal marker *Xbra* and the posterior marker *Xcad3*, in CS2-*Xwnt8* injected embryos. Strong upregulation of the expression of *Hoxd1* was observed (Fig. 4.5A). Not only is the expression domain larger as compared to control embryos, but the

expression also appears earlier and can be observed in the organiser field (Fig. 4.5A). Later during gastrulation, ectopic *Hoxd1* expression continues to be present in ectoderm and mesoderm in the midline of the embryo, and is expanded anteriorly (Fig. 4.5A). *Hoxa1* is upregulated in the gastrula and its horseshoe pattern closes over the organiser to form a ring, after *Xwnt8* overexpression; at stage 13 however, *Hoxa1* expression is no longer upregulated (Fig. 4.5B). *CS2-Xwnt8* injections cause strong ectopic upregulation of *Hoxb1* at both stage 11 and stage 13 (Fig. 4.5C); upregulation appears randomly distributed throughout the embryo and is observed in both ectoderm and mesoderm, with considerable variability between embryos (Fig. 4.5C shows one example). Expression of *Hoxb4* is upregulated in a similar way to *Hoxa1* in the early gastrula (stage 11) and, like this latter gene, it is no longer upregulated at stage 13 (Fig. 4.5D) by *Xwnt8* gain-of-function (Fig. 4.5B). Expression of *Hoxd4* (Fig. 4.5E) is upregulated during gastrula as well as neurula stages. *Wnt8* gain-of-function causes ectopic and premature expression of *Hoxc6* in dorsal (organiser) mesoderm, a tissue that normally does not express *Hox* genes. Later in gastrulation, an expansion of the endogenous horseshoe-shaped domain is observed (data not shown). In early neurula stages, an anterior expansion of the expression of *Hoxc6* is observed in the neuroectoderm (Fig. 4.5F). The effect of *Xwnt8* injection on *Otx2* expression is the opposite of what is seen for most *Hox* genes: it is downregulated (Fig. 4.5G). Expression of the mesodermal marker *Xbra* is unaltered by *Xwnt8* (Fig. 4.5H), suggesting that mesoderm formation is not affected. Expression of the posterior marker *Xcad3* is upregulated in mesoderm (data not shown) and ectoderm of

injected embryos (Fig. 4.5I), confirming the posteriorising nature on neuroectoderm of CS2-*Xwnt8* injection. The different effects of misexpression of *Xwnt8* on *Hoxd1 Hoxa1*, *Hoxb4*, *Hoxd4* and *Hoxc6* demonstrates the complex and dynamic regulation of *Hox* gene expression in marginal zone mesoderm, and suggests a role for Wnt signalling in this regulation. These results were extended by using RT-PCR to monitor *Hox* expression (Fig. 4.6). This confirmed the effects of *Xwnt8* ectopic expression on *Hoxd1*, *Hoxa1, Hoxb1* and *Hoxb4* (only detected strong enough at st. 13) and *Hoxc6* as well as showing that *Hoxa7* is upregulated at both st. 11 and 13. *Hoxb5* was not visibly affected, while *Hoxc8* appeared downregulated (Fig. 4.6).

**Labial type *Hox* genes are direct targets of canonical Wnt signalling (in collaboration; P. in der Rieden)**

It is generally believed that *Xwnt8* acts mainly through the canonical Wnt pathway before and after the onset of gastrulation, stabilizing cytosolic β-catenin and activating gene expression through Tcf/Lef transcription factors (Darken and Wilson, 2001). To investigate whether the induction of anterior *Hox* genes by canonical Wnt signalling is direct we made use of an activated, hormone inducible form of *XTcf3*, TVGR (Darken and Wilson, 2001). Embryos were injected into the animal hemisphere at the one-cell stage with 100 pg of TVGR. Cycloheximide (CHX) was added at stage 9.5, followed half an hour later by dexamethasone (DEX), well before the onset of gastrulation and the initiation of *Hox* gene expression. At stage 11 the embryos were harvested for RT-PCR. The results are shown in figure 6. Because CHX was added before the onset of gastrulation, induction of

*Hoxd1*, *Hoxa1, Hoxb1, Hoxb4* and *Hoxc6* expression in control embryos is reduced or absent (Fig. 4.7). Expression of *Hoxd1*, *Hoxa1* and *Hoxb1* is directly activated by the TVGR, while none of the other *Hox* genes examined is induced. (Fig. 4.7). The expression of *Hoxb4, Hoxc6* and *Hoxa7* is however upregulated when DEX was added in the absence of CHX, suggesting that induction of expression of these more posterior genes is indirect.

In situ hybridisation (Fig. 4.8) confirmed the results obtained by RT-PCR. Whereas injection of TVGR does not significantly affect normal expression of *Hoxd1*, *Hoxb1* and *Hoxa1* (*Hoxc6* transcript levels were somewhat downregulated), co-addition of CHX TVGR strongly reduces the intensity of expression, and in some cases also the size of the expression domain. Induced expression *Hoxa1* and *Hoxd1* following activation of TVGR is confined to the usual NOM mesodermal domain, while expression following TVGR and CHX is not: it extends into the organiser mesoderm and into the ectodermal animal cap. This suggests that CHX blocks the production of repressors that normally contribute to define the limits of the most anterior (thus neighbouring the organiser domain) *Hox* expression domains. *Hoxb1*, on the other hand, is ectopically upregulated in different parts of the embryo, but its scattered and the relatively low level of upregulation makes it difficult to determine whether this takes place within or outside the normal *Hox* expression domain. *Hoxd4* behaves very similarly to *Hoxc6* under all conditions (data not shown). Together, these results indicate that activation of the canonical Wnt pathway around the time of gastrulation directly induces expression of anterior (paralogue 1) group *Hox* genes.

## Discussion

### Ectopic *Xwnt8* directly initiates expression of *Hoxa1, Hoxb1* and *Hoxd1*

We report that ectopic *Xwnt8 expression* can initiate *Hoxd1*, *Hoxa1* and *Hoxb1* expression in mesoderm and ectoderm of *Xenopus* gastrula stage embryos. *Xwnt8* is thus able to induce expression of the three earliest expressed *Hox* genes in *Xenopus*, earlier than initiation of endogenous expression and in tissues normally not expressing these *Hox* genes as well as in endogenously expressing tissues. Kiecker and Niehrs (2001) have reported that the injection of pCSKA-*Xwnt8* (CSKA-X8, Christian and Moon, 1993) into *Xenopus* embryos does not alter the expression of *Hoxd1*, in contrast to the results presented here. In our hands pCSKA-*Xwnt8* was also not able to initiate the expression of *Hoxd1* in mesoderm or ectoderm. This could be due to the specific UTR sequences contained in the pCSKA-*Xwnt8* plasmid, as UTR sequences are known to affect the stability of mRNA and to regulate the translation of the messenger (reviewed in Derrigo *et al.*, 2000, and references therein).

We show that *Xwnt8* can initiate the expression of *Hoxd1*, *Hoxa1* and *Hoxb1* and that induction of these three genes by the canonical Wnt pathway is direct. Activation of a hormone inducible VP16 (constitutively active) form of the transcription factor *Xtcf3*-TVGR (Darken and Wilson, 2001) induces expression of *Hoxd1*, *Hoxa1* and *Hoxb1* in the presence of the protein synthesis inhibitor cycloheximide*. Hoxa1*, *Hoxd1* and *Hoxb1* were the only three of 7 *Hox* genes examined to be regulated directly. Other *Hox* genes (*Hoxa7, Hoxc6* and *Hoxd4*) were regulated only indirectly and *Hoxb4* did not appear to be responsive to *Wnt*.

**Endogenous *Xwnt8* signalling is necessary for endogenous expression of *Hoxa1*, *Hoxd1* and *Hoxb1*, as well as other *Hox* genes, in dorsolateral mesoderm and neuroectoderm**

*Xnwt8* loss-of-function experiments suggest that *Xwnt8* is required for *Hoxa1, Hoxd1* and *Hoxb1* expression in marginal zone mesoderm. A mechanism whereby different inputs are capable of starting *Hox* expression from different *Hox* paralog groups or from different *Hox* clusters could be of importance in the regulation of *Hox* gene expression and thus for patterning the anteroposterior axis. The effect of *Xwnt8* loss-of-function on expression of *Hoxc6* expression is striking. *Hoxc6* is upregulated in dorsal mesoderm and ectoderm, tissues normally not expressing *Hoxc6*, significantly earlier than endogenous expression. This result was confirmed by multiple experiments using both in situ hybridisation and RT-PCR, but it is difficult to understand. A partial explanation could lie in the fact that *Wnt*s are known to cause repression as well as activation. Perhaps we are concerned here with a balance between activation and repression on the same gene such that while normal levels do not induce it, very high or very low ones do. The necessity of Wnt signalling for the expression of labial-type *Hox* genes is also supported by findings in *C. elegans* (Streit *et al.*, 2002), where expression of *ceh-13*, the nematode *labial* ortholog, depends on *Wnt* signalling. Strikingly, regulatory elements of *ceh-13* can act as Wg response elements in transgenic *Drosophila* embryos. Together with our results, this evidence points to a conserved and ancient mechanism wherein labial-type *Hox* gene expression is dependent on *Wnt* signalling. To our knowledge it

has never previously been reported that labial-type *Hox* genes can be induced by *Wnt* signalling in the absence of protein synthesis.

**The early role of *Xwnt8***

Our findings above indicate a specific early role for *Xwnt8* during gastrulation. It directly activates expression of the three earliest expressed labial *Hox* genes (*Hoxd1*, *Hoxa1* and *Hoxb1*) and consecutively it possibly initiates expression of the *Hoxa*, *Hoxd* and *Hoxb* clusters. In agreement with this, *Xwnt8* activates expression of other *Hox* genes, but only indirectly. The specificity of these effects is emphasised by the fact that not all *Hox* genes examined are strongly regulated by *Xwnt8* in the early embryo, even though all of these genes are expressed. This appears to be the first report of a direct initiation factor necessary for early expression of *Hox genes* in the early embryo. Presumably, *Wnt8* also induces other factors needed for progression. One factor that could be involved is *Raldh2* (enzyme responsible for the synthesis of retinoic acid), which is strongly induced by the *Wnt* pathway (own observations). Retinoid signalling is, in turn, known to regulate neuroectodermal expression of 3' anterior *Hox* genes (Lumsden and Krumlauf, 1996; Durston et al., 1998; Godsave et al., 1998; Bel-Vialar et al., 2002). It is also known that knocking out all three labial *Hox* genes causes loss of expression of other *Hox* genes in the early neural plate. Altogether, these factors could act in parallel to coordinate an appropriate *Hox* gene expression profile in the early embryo, which is crucial for the overall patterning of the anterior-to-posterior axis.

**Is *Xwnt8* involved in generating a gradient?**

The different effects of *Xwnt8* function on the expression of different *Hox* paralog groups and *Hox* clusters may contribute to the generation of an early *Hox* pattern. This pattern is initiated in mesoderm, followed by the appearance of the *Hox* sequence in neuroectoderm. A posterior to anterior positive gradient of β–cat/Wnt signalling in neuroectoderm has previously been postulated to underlie the embryo's neuroectodermal A-P pattern (Kiecker and Niehrs, 2001). In fact, there are now many observations that make the action of one or more *Wnt* gradients likely in the AP patterning of the developing central nervous system. These range from gradients acting within the anterior brain (Lagutin et al., 2003) through patterning of the fore-mid-hindbrain region (Nordström et al., 2002) and distinguishing between head and trunk to patterning of the posterior CNS (Nordström et al., 2006). Some of the findings feature interactions between *Wnt* and other pathways, with *Wnt* being a source of graded information and other pathways sometimes being permissive.

The following point of contact with our own observations is very interesting. Nordström et al. (2006) documented the responses of three *Hox* genes to Wnt3A protein. These are all *Hoxb* genes, representing different positions along the posterior neuraxis. These are however responses of gastrula neural plate explants to long periods (up to 44 hours) of exposure to WNT protein, leading to a much later analysis of *Hox* expression. The *Hox* gene response also requires FGF and, in the case of *Hoxb4*, retinoic acid. This study differs from our own in several respects. It is in chicken, the response is to Wnt3A, and it examines exclusively a neural plate cell population (and

not the earliest mesodermal *Hox* expression population). It is likely that the last parameter is a crucial one.

In our own study, the very early responses of *Hox* genes seem not to fit the idea of a gradient of *Wnt8*. We are concerned with gastrula stages, where A-P patterning genes (*Hox* genes and *Otx2*) are already expressed, but where the A-P axis is not yet obviously set up. At this stage, the information for the A-P *Hox* sequence seems to be contained in a temporally collinear sequence of *Hox* expression in the gastrula non-organiser mesoderm. If a gradient of anteroposterior patterning information were to spread from the future posterior tissues to future more anterior tissues, we expect the *Hox* genes to be functional downstream of this gradient and, as a consequence, to respond to changes in it. The observed effects on *Hox* expression by *Xwnt8* loss- and gain-of-function make the existence of such an early gradient unlikely or at least, argue against regulation of all of these *Hox* genes by thresholds on an early source of WNT signal. In *Xwnt8* loss-of-function, posterior *Hox* genes would be expected to be downregulated, considering that the source of the gradient is thought to be in the posterior part of the embryo. This is in conflict with the observed induced expression of *Hoxc6* in dorsal mesoderm and ectoderm, and with an enhanced level of *Hoxc6* expression in ventrolateral mesoderm of embryos injected with $MO^{Xwnt8}$.

The differential effects of early *Xwnt8* loss- and Wnt gain-of-function on expression of *Hoxa1*, *Hoxd1* and *Hoxb1* are also not consistent with a simple model wherein a gradient of *Wnt* signalling along the anteroposterior axis is used to provide positional information within the trunk (*Hox*

expressing) part of the axis. According to such a model, a gene expressed more anteriorly could be expressed in more posterior tissues in response to loss-of-function for the gradient. The results of MO$^{Xwnt8}$ experiments contradict this idea; they show a strong downregulation of *Hoxd1* expression in embryos with reduced *Xwnt8* signalling, and never a posterior expansion of the *Hoxd1* expression zone. Upregulation of *Hoxc6* expression observed in *Xwnt8* gain-of-function is much weaker and in a significantly smaller domain than upregulated *Hoxd1* expression, while leaving expression of *Hoxb4* virtually unaffected. These results also contradict a model whereby an anteroposterior gradient of *Wnt* signalling is used to pattern the early primary axis. We propose a model wherein *Xwnt8* is involved in initiating a pattern of *Hox* expression in the ventrolateral marginal zone mesoderm of the embryo by initiating the expression of *Hoxd1* and *Hoxa1*, in a direct fashion. After the initial activation, the *Hox* cascade continues, by progressive, temporally collinear opening and expression of the *Hox* clusters. *Hox* genes from other paralogue groups are induced indirectly because they depend on *Wnt* initiation of *Hox* cluster opening but also require other factors than just *Wnt* signalling, thereby creating steps in the *Hox* code. This leads to our conclusion that *Xwnt8,* and perhaps other *Wnts*, play an important part in setting up the early *Hox* codes. In fact this code of collinearly expressed *Hox* genes can be considered as a map of positional information along the anteroposterior axis. We note that there is evidence for interactions among *Hox* genes in the early embryo. Most notably, simultaneous MO loss of function of all three *Hox*1 (labial *Hox*) genes anteriorises the most anterior *Hox* expressing part (hindbrain region) of the

early neuraxis, deleting expression of all *Hox* 1, 2 and 3 paralogues and deleting the anterior parts of the expression domains of *Hox* 4, 5 and 6 paralogues. The anterior part of the neural plate that normally makes the hindbrain is converted to the identity of rhombomere 1 (expressing *Gbx2*).

***Figure 4.1.*** **Expression of *Xwnt8* during gastrula and early neurula stages.** Embryos were assayed for expression of *Xwnt8* by whole-mount *in situ* hybridisation. In each panel a single embryo is shown. **(A)** Stage 11 embryo, lateral view with dorsal to the left, and a sagittal section of the embryo. *Xwnt8* expression is detected in the ventral and lateral marginal zone mesoderm. **(B)** Stage 11.5 embryo, lateral view with dorsal to the left, and a lateral to lateral section section. Expression can be found close to the blastopore and in involuted mesoderm. **(C)** Stage 12 embryo, lateral view with dorsal to the left, and a posterior view. Expression of *Xwnt8* can be found in presumptive paraxial mesoderm and expression close to the blastopore is further restricted to dorsolateral positions. **(D)** Stage 13 embryo, lateral view with anterior to the left, a posterior view of the embryo, and two transverse sections. In the section on the right top of the panel *Xwnt8* expression in presumptive hindbrain is shown, this corresponds to the most anterior expression in the lateral view. Expression in mesoderm close to the closing blastopore is shown in the bottom right section of the panel and corresponds to expression shown in posterior view. **(E)** Stage 17 embryo, lateral view with anterior to the left, posterior view, and a transverse section. The anterior ectodermal expression domain, the paraxial expression, and the dorsolateral expression in the mesoderm remain, while a lateral expression domain appears in the ectoderm. In the dorsal-to-ventral section, and in an enlargement on the bottom right of the panel, initiation of *Xwnt8* expression in the neural tube can be found. (in collaboration; P. in der Rieden).

***Figure 4.1***

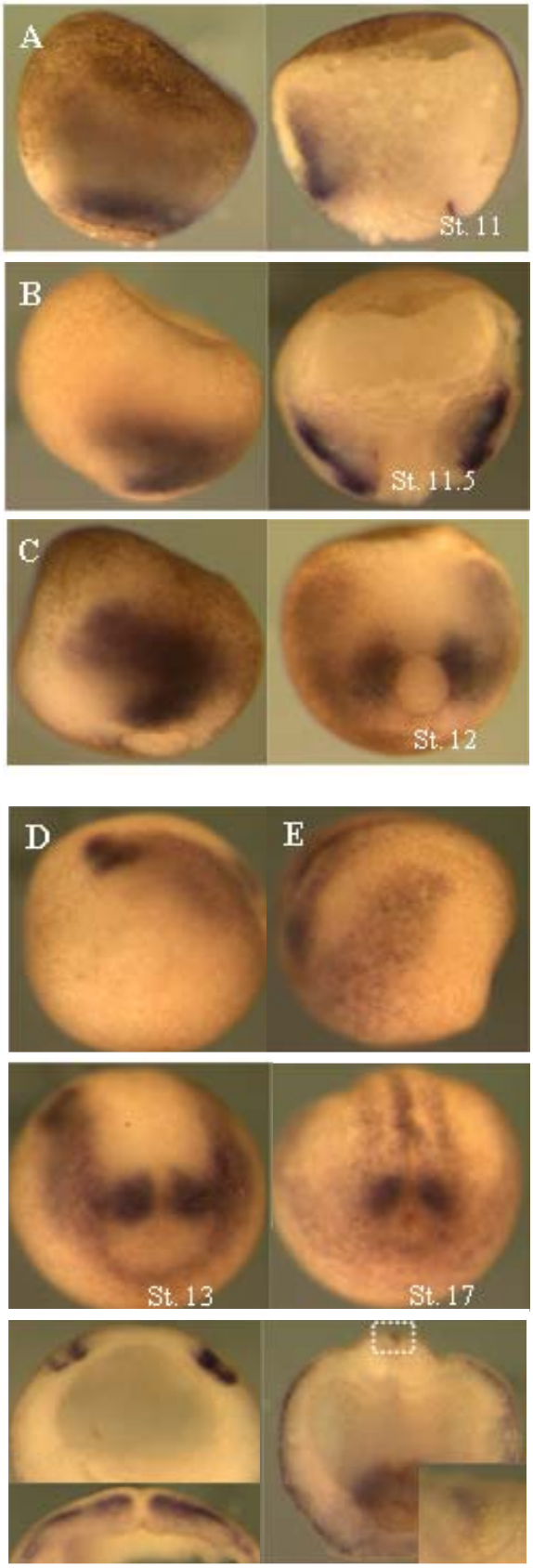


***Figure 4.2.*** **Expression of *Xwnt8*, *Hoxd1*, *Hoxb4*, and *Hoxc6* during gastrulation.** Embryos were analysed by whole-mount *in situ* hybridisation for expression of *Xwnt8* **(A)**, *Hoxd1* **(B)**, *Hoxb4* **(C)**, and *Hoxc6* **(D)**. Embryos are shown, going from left to right through the panels, at stage 11, stage 11.5, stage 12 (vegetal views with dorsal up), and at stage 13 (vegetal views with dorsal up). *Xwnt8* expression overlaps with the expression of *Hoxd1* in the ventrolateral mesoderm during early gastrulation. At stage 12 the posterior most expression of *Xwnt8* becomes restricted to dorsolateral marginal zone, overlapping with the expression domain of *Hoxd1*. When gastrulation is nearly completed an overlap in expression of *Xwnt8* and *Hoxd1* can be observed in presumptive hindbrain, and paraxial mesoderm. *Hoxb4* and *Xwnt8* show an overlap in their expression patterns during stage 11.5, at stage 12 ectodermal expression of *Hoxb4* is initiated in overlapping the dorsolateral *Xwnt8* expression domain. During late gastrulation an overlap in expression of *Hoxb4* and *Xwnt8* is observed in paraxial mesoderm. Expression of *Hoxc6*, on the other hand, is initiated after the retraction of the *Xwnt8* expression to the dorsolateral domains, therefore an overlap in expression is only observed there. This overlap is still visible at the end of gastrulation. Likewise for *Hoxd1* and *Hoxb4* the first ectodermal expression is initiated of *Hoxc6* found in the ectoderm overlying the posterior dorsolateral domains of *Xwnt8* expression. (in collaboration; P. in der Rieden)

***Figure 4.2***

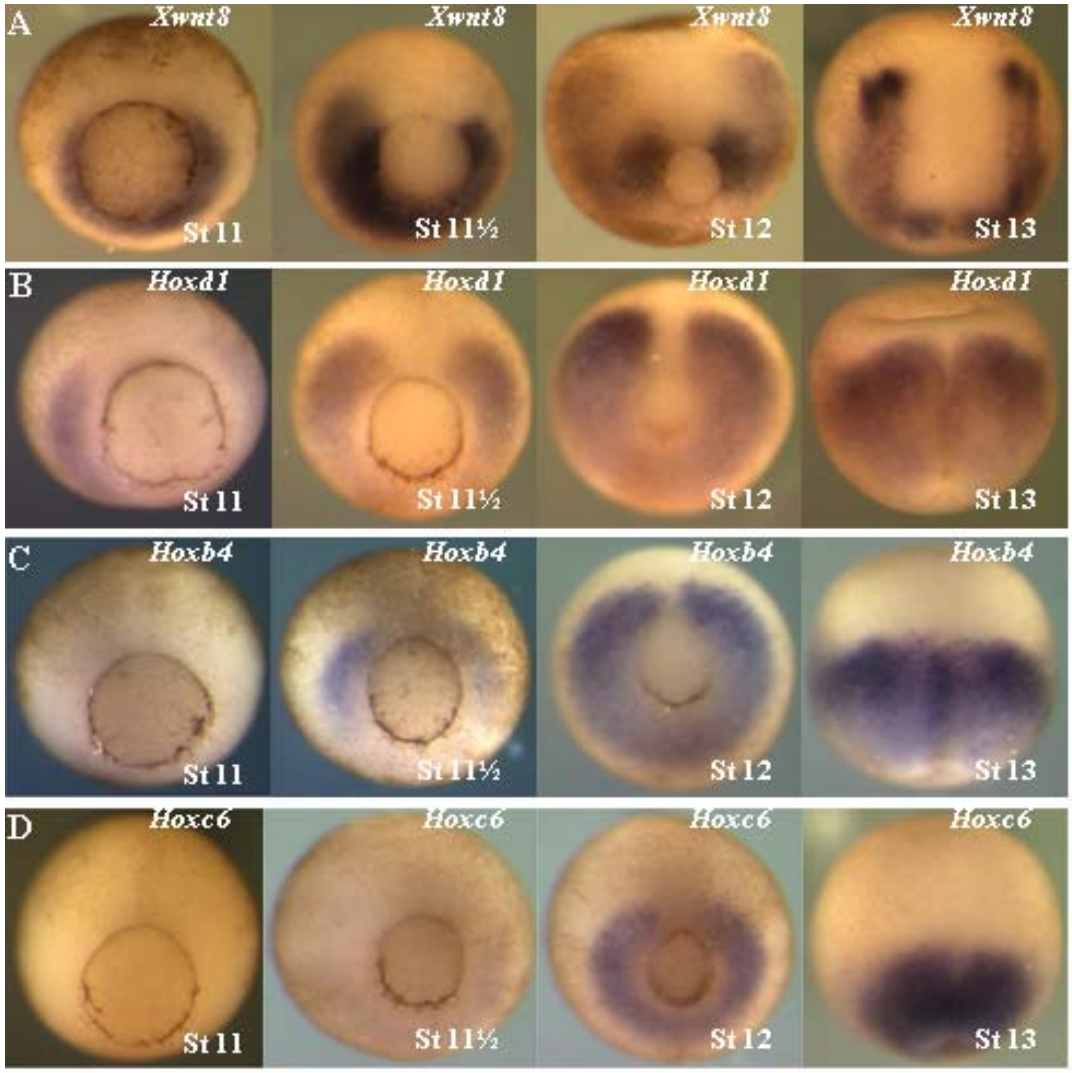

A
Xwnt8
St 11
Xwnt8
St 11½
Xwnt8
St 12
Xwnt8
St 13
B
Hoxd1
St 11
Hoxd1
St 11½
Hoxd1
St 12
Hoxd1
St 13
C
Hoxb4
St 11
Hoxb4
St 11½
Hoxb4
St 12
Hoxb4
St 13
D
Hoxc6
St 11
Hoxc6
St 11½
Hoxc6
St 12
Hoxc6
St 13

***Figure 4.3*. Effects of *Xwnt8* loss-of-function on phenotype and rescue of MO*Xwnt8***. Embryos at the one-cell stage were injected into the animal hemisphere with 64 ng of MO*Xwnt8*, and allowed to develop until the control embryos reached stage 24 **(A)** or stage 35 **(B)**. In the majority of the embryos the axis is reduced and the head is enlarged, as is the most anterior structure, the cement gland: (extreme form) **(D)** and (moderate form) **(C** and **E)**. **(F)** The specificity of the MO*Xwnt8* is shown by rescue with MOI CS2*Xwnt8* plasmid. Embryos were injected with 20 pg MOI CS2*Xwnt8,* 64 ng MO*Xwnt8*, or with both, and then compared to non-injected embryos. (in collaboration; P. in der Rieden).

***Figure 4.3***

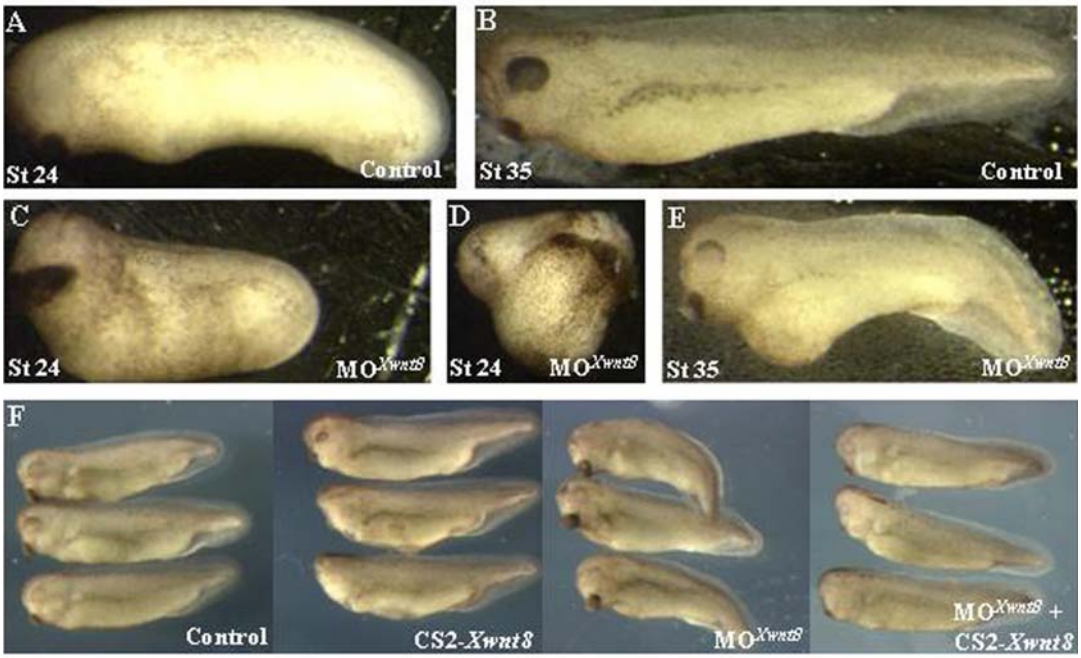
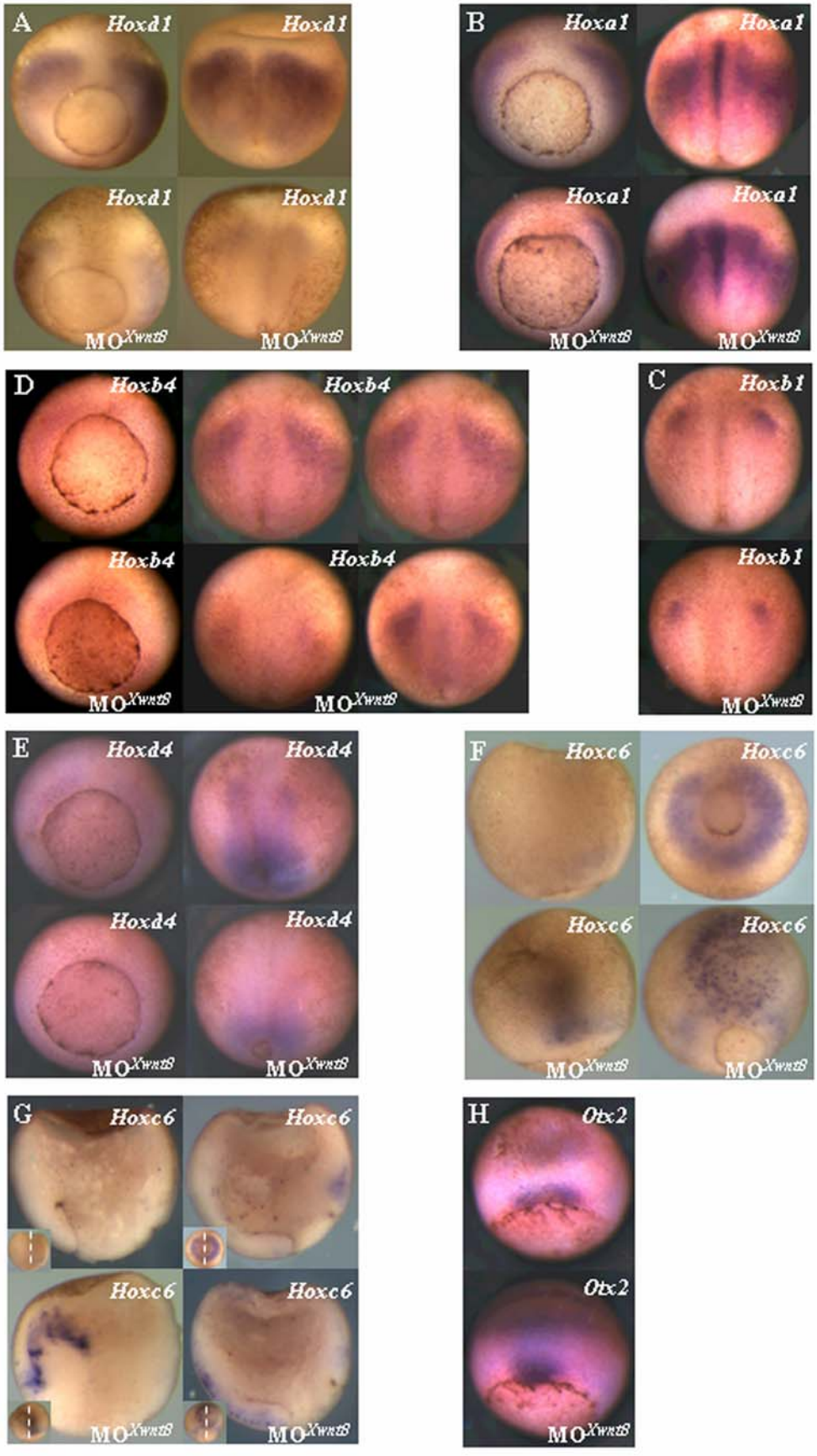

A
St 24
Control
B
St 35
Control
C
St 24
MO^Xwnt8
D
St 24
MO^Xwnt8
E
St 35
MO^Xwnt8
F
Control
CS2-Xwnt8
MO^Xwnt8
MO^Xwnt8 +
CS2-Xwnt8

***Figure 4.4.*** **Effects of *Xwnt8* loss-of-function on expression of *Hoxd1*, *Hoxa1, Hoxb1, Hoxb4*, *Hoxd4* and *Hoxc6,* as well as *Otx2*.** Embryos were injected into the animal hemisphere at 2-cell stage with 32 ng of MO*Xwnt8* per cell and analysed by whole-mount *in situ* hybridisation. Injected embryos are shown at the bottom of each panel, control embryos are shown on top. Shown are vegetal views with dorsal to the top. (A) Expression of *Hoxd1* is downregulated by MO*Xwnt8* injections, shown are stages 11 (left side of the panel), and stage 12 (right side of the panel). (B) Expression of *Hoxa1* is also downregulated by the MO*Xwnt8* at stage 11 (left side of the panel), but it isn't visibly affected at stage 13 (right side of the panel). (C) *Hoxb1* presents a shrinking expression domain upon loss-of-function at stage 13. (D) Expression of *Hoxb4*, shown at stage 11 (left side of the panel) and stage 13 (right side of the panel), is downregulated in some embryos by *Xwnt8* loss-of-function, although in other cases they appear unaffected (see one example of each at st. 13). (E) *Hoxd4* is downregulated by the MO*Xwnt8*; shown are stages 11 (left side of the panel) and 13 (right side of the panel). (F) Expression of *Hoxc6* is upregulated by *Xwnt8* loss-of-function on the dorsal side of the embryo; shown are stages 10.5 (left side of the panel) and 11.5 (right side of the panel). Dorsal to ventral sections of the embryos shown in (G); the plane of sectioning is depicted by the dotted line in the insets on the bottom left corner. (H) Finally, *Otx2* expression is increased by MO*Xwnt8* injections at stage 10.5 (embryos were slightly turned so that the dorsal expression domain could be better seen; the blastopore remains at the bottom).

***Figure 4.4***


A
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Hoxd1
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Hoxa1
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Hoxb4
Hoxb4
Hoxb4
MO Xwnt8
MO Xwnt8
C
Hoxb1
Hoxb1
MO Xwnt8
E
Hoxd4
Hoxd4
Hoxd4
Hoxd4
MO Xwnt8
MO Xwnt8
F
Hoxc6
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MO Xwnt8
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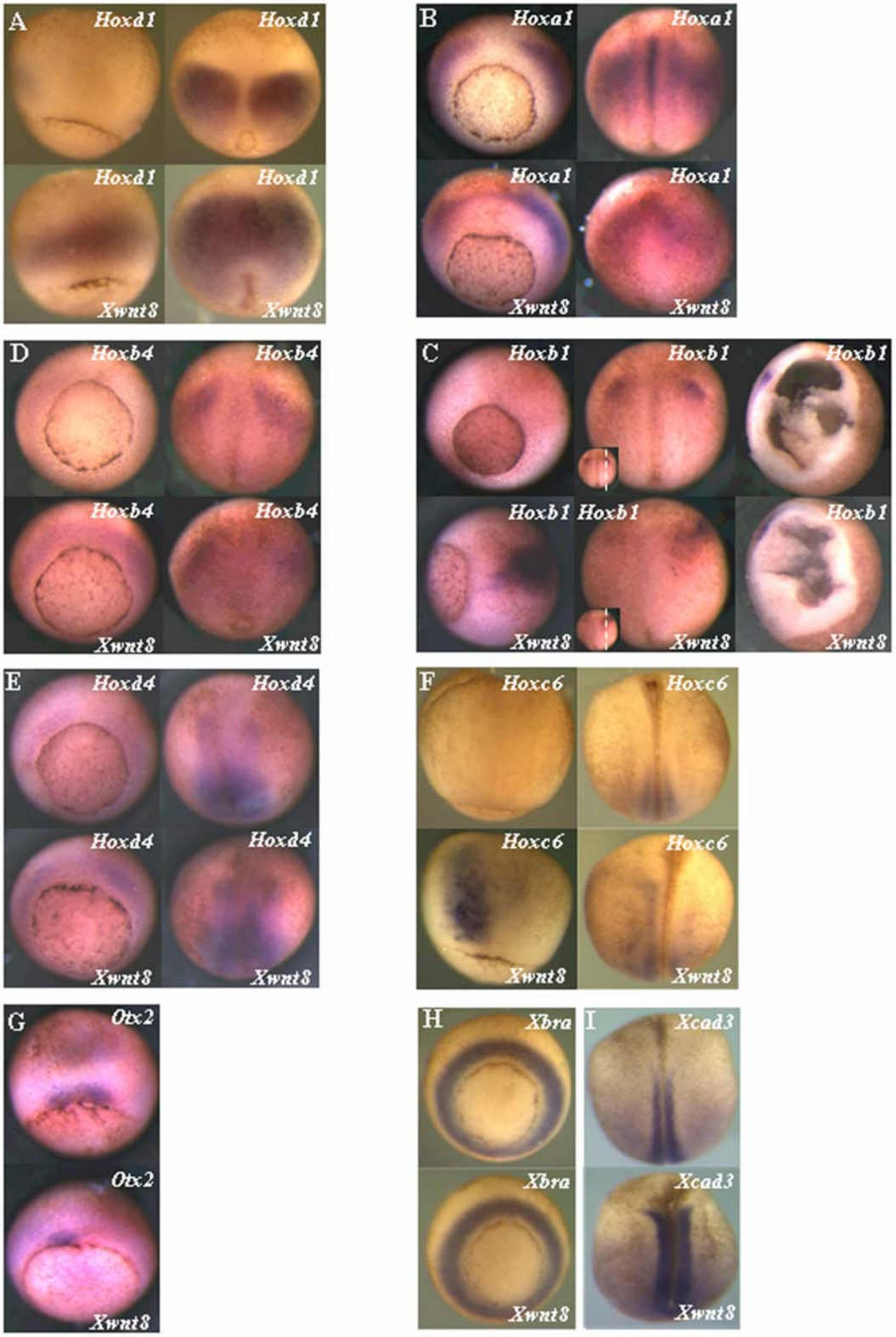
G
Hoxc6
Hoxc6
Hoxc6
Hoxc6
MO Xwnt8
MO Xwnt8
H
Otx2
Otx2
MO Xwnt8

***Figure 4.5.*** **Effects of *Xwnt8* gain-of-function on the expression of *Hoxd1*, *Hoxa1*, *Hoxb1*, *Hoxb4*, *Hoxd4*, *Hoxc6*, as well as *Xbra*, *Xcad3* and *Otx2*.** Embryos were injected at the one-cell stage into the animal hemisphere with 100 pg CS2-*Xwnt8* plasmid, and analysed by whole-mount ISH. Injected embryos are shown on the bottom of each panel, control embryos on the top. (A) Expression of *Hoxd1* is ectoptically upregulated in dorsal tissues of injected embryos; shown are stage 10 (left side of the panel) and stage 12.5 (right side of the panel) embryos, the views are dorsal with anterior to the top. (B) *Hoxa1* expression is upregulated at stage 11 and the horseshoe pattern closes up in the dorsal midline of the embryo after overexpression of *Xwnt8* (left side of the panel); at stage 13 there is no evident alteration of expression (right side of the panel); views are vegetal with dorsal to the top. (C) *Xwnt8* injections lead to a strong ectopic expression of *Hoxb1* at both stage 11 (left side of the panel) and stage 13 (middle column of the panel); views are vegetal with dorsal to the top, except for the stage 11 injected embryo, which is seen from the lateral. Dissections of the stage 13 embryos are presented (left side of the panel); embryos were cut along the anterior-to-posterior axis, as depicted by the dotted lines in the insets on the bottom left corner. (D) The expression of *Hoxb4* is upregulated at stage 11 and the horseshoe pattern closes up on the dorsal midline of the embryo after *Xwnt8* gain-of-function (left side of the panel); expression is not obviously changed at stage 13 (right side of the panel); views are vegetal with dorsal to the top. (E) *Hoxd4* expression becomes stronger after gain-of-function, shown are stages 11 (left side of the panel) and 13 (right side of the panel); views are vegetal with dorsal to the top. (F) Expression of *Hoxc6* is upregulated dorsally at stage 10 (left side of the panel), and in neuroectoderm of stage 15 embryos (right side of the panel). (G) *Otx2* is downregulated by the overexpression of *Xwnt8*, shown are stage 10.5 embryos with the blastopore down and the dorsal side to the top. (H) Expression of the mesodermal marker *Xbra* appeared unaltered; shown are stage 11 embryos in vegetal view with dorsal up. (I) Finally, expression of the posterior marker *Xcad3* is shifted to a more anterior position as a result of the *Xwnt8* gain-of-function; shown are embryos at stage 17, dorsal view with anterior up. (in collaboration; P. in der Rieden)

***Figure 4.5***


A
Hoxd1
Hoxd1
Hoxd1
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Xwnt8
Xwnt8
B
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Hoxa1
Hoxa1
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Xwnt8
Xwnt8
D
Hoxb4
Hoxb4
Hoxb4
Hoxb4
Xwnt8
Xwnt8
C
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Hoxc6
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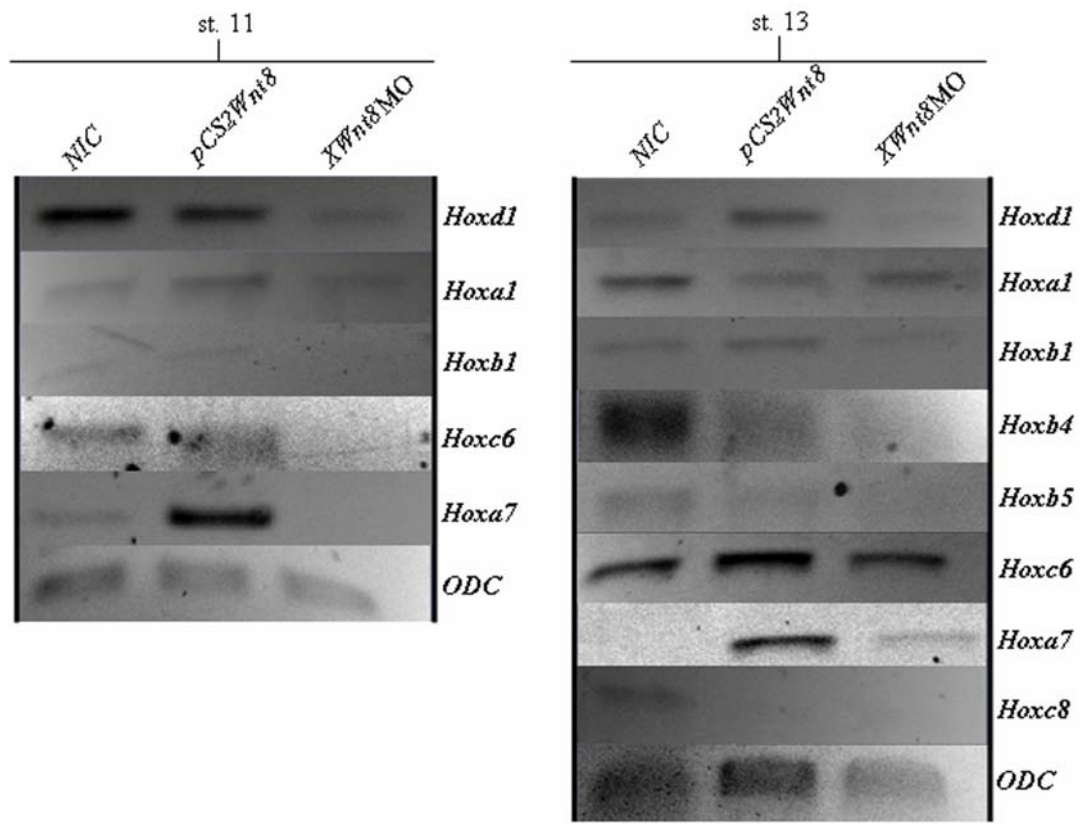
Hoxc6
Hoxc6
Xwnt8
Xwnt8
G
Otx2
Otx2
Xwnt8
H
Xbra
Xbra
Xwnt8
I
Xcad3
Xcad3
Xwnt8

***Figure 4.6.*** **Effects of *Xwnt8* gain (GOF) and loss (LOF) of function on the expression of several *Hox* genes.** Embryos were injected at the 2-cell stage with either 50 pg CS2*Xwnt8* DNA into each cell for GOF or 32 ng *Xwnt8* MO into each cell for LOF. Total mRNA was collected from either st. 11 or st. 13 embryos. Each row displays an agarose gel slice loaded with the products of RT-PCR for the gene noted on the right hand side. *ODC* is used as a loading control. Conditions are writen on the top.

***Figure 4.6***


st. 11
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XWnt8MO
Hoxd1
Hoxa1
Hoxb1
Hoxc6
Hoxa7
ODC
st. 13
NIC
pCS2Wnt8
XWnt8MO
Hoxd1
Hoxa1
Hoxb1
Hoxb4
Hoxb5
Hoxc6
Hoxa7
Hoxc8
ODC

***Figure 4.7.*** Tcf/Lef signalling is directly upstream of expression of *Hoxd1*, *Hoxa1* and *Hoxb1*. Embryos at the one-cell stage were injected into the animal hemisphere with 100 pg TVGR, an activated hormone inducible form of *XTcf3*. Cycloheximide (CHX) was added before the start of gastrulation, followed by addition of dexamethasone (DEX), see for details the materials and methods section. In control embryos expression of *Hoxd1*, *Hoxb4* and *Hoxc6* was repressed or inhibited by addition of CHX (notice that both *Hoxa1* nad *Hoxb1* expreession levels are too low at that stage in NIC); addition of DEX on the other hand did not lead to a difference in expression of the five *Hox* genes assayed; expression in the combined CHX and DEX treatment appears as in the only CHX treatment. Injection of TVGR and subsequent addition of CHX strongly downregulates expression of *Hoxb4* and *Hoxc6*. Activation of TVGR by DEX however, led to an induction of *Hoxd1*, *Hoxa1* and *Hoxb1* expression. The induction of *Hoxd1*, *Hoxa1* and *Hoxb1* are shown to be direct by addition of DEX in presence of CHX, whereas expression of *Hoxb4* and *Hoxc6* after DEX is drastically repressed by co-addition of CHX and is therefore indirect. Notice: the last lane from the top, labelled with *ODC,* is the loading control for *Hoxa1* and *Hoxb1*; the fourth lane, also labelled with *ODC*, corresponds to *Hoxd1*, *Hoxb4* and *Hoxc6*. This is a result of those being two separate experiments. (in collaboration; P. in der Rieden)

***Figure 4.7***

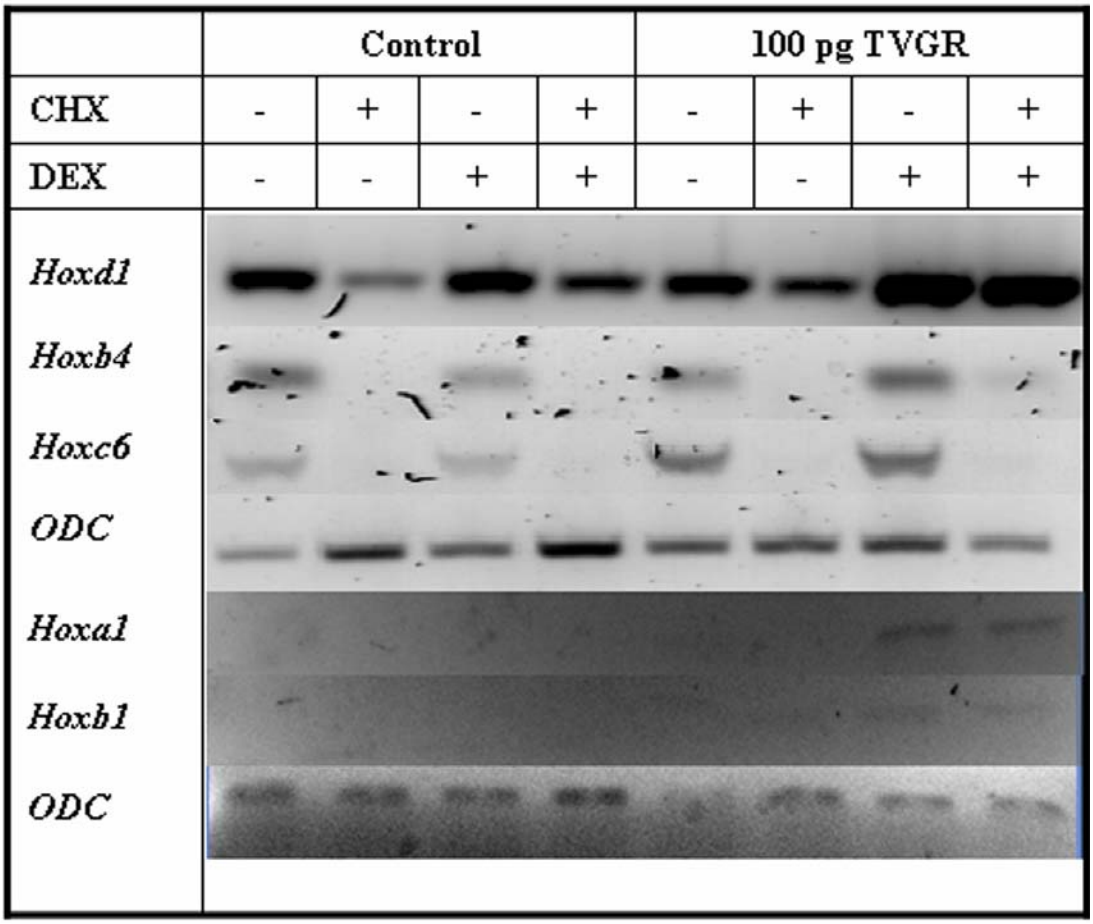
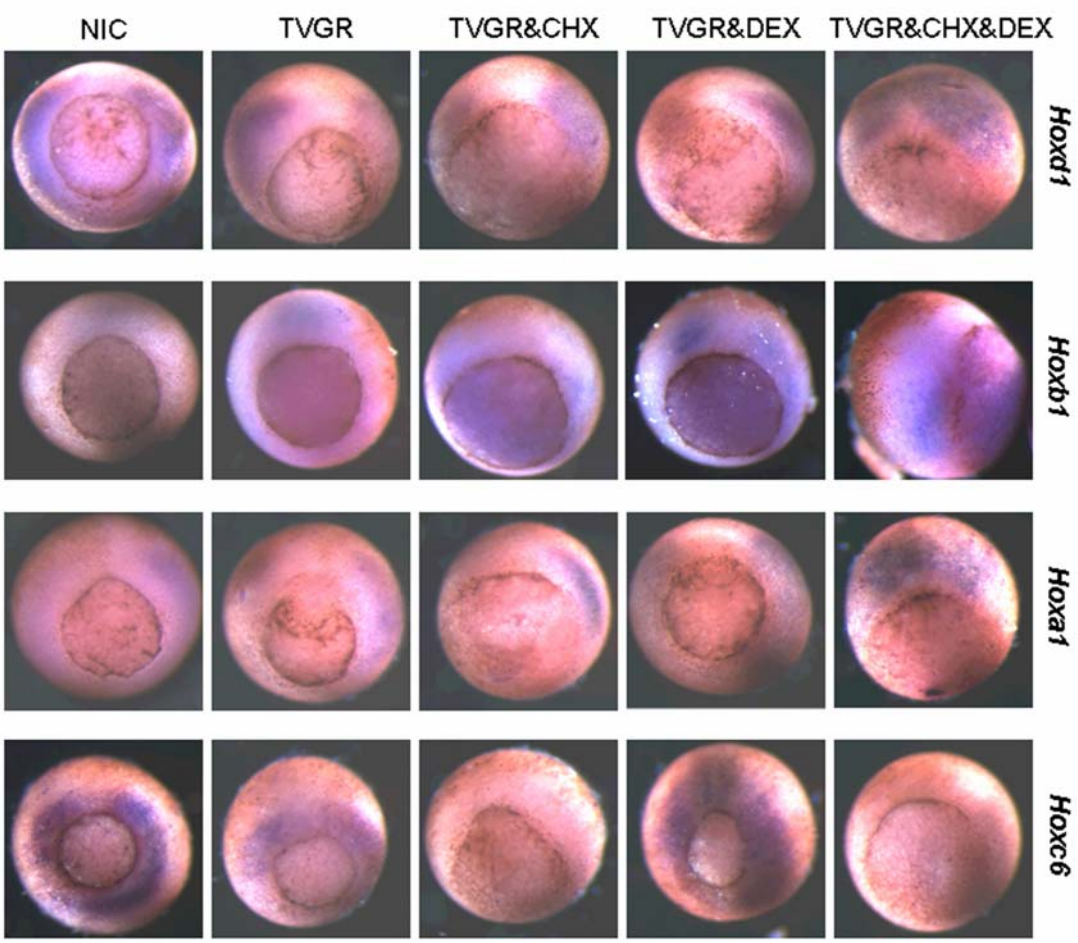

Control
100 pg TVGR
CHX
DEX
- + - + - + - +
- - + + - - + +
Hoxd1
Hoxb4
Hoxc6
ODC
Hoxa1
Hoxb1
ODC

***Figure 4.8.*** **Different effects of TVGR (a hormone inducible form of *XTcf3*) and its subsequent activation on the expression of four *Hox* genes.** Embryos were injected at the 2-cell stage with 50 pg of TVGR DNA into each cell; before gastrulation CHX was added to prevent protein synthesis, followed by co-addition of DEX in order to trigger activation of the hormone inducible system. Embryos were fixed after the standard treatment (see Materials and Methods). Each row shows in situ hybridisations with probes for the respective genes noted on the right hand side; each column corresponds to the conditions described on top of it. Injection of TVGR doesn't significantly alter the normal (NIC) expression pattern of these genes, except for *Hoxb1*, which shows some ectopic expression. Addition of DEX in injected embryos causes substantial upregulation of *Hoxd1*, *Hoxb1*, *Hoxa1* and *Hoxc6*. Co-addition of CHX and DEX in TVGR injected embryos triggers a massive induction of both *Hoxd1* and *Hoxa1*, as well as induction of *Hoxb1*; under these conditions, *Hoxc6* upregulation caused by DEX alone on TVGR injected embryos is abolished. All views are vegetal.

***Figure 4.8***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TVGR&CHX&DEX
Hoxd1
Hoxb1
Hoxa1
Hoxc6

**Chapter 5**

# Homeoproteins as intercellular messengers in the early *Xenopus laevis* embryo

## Introduction

Evidence so far suggests that initial establishment of *Hox* expression in the vertebrate embryo is tissue specific, with different factors and/or morphogens involved in patterning the non-organiser (paraxial and ventrolateral) mesoderm and the neuroectoderm (neural plate) (the present work). Wnt signalling acts in the mesoderm of *Xenopus laevis* early gastrula to induce *labial*-type *Hox* genes (*Hoxd1*, *Hoxa1* and *Hoxb1*) in a direct fashion, independent of protein synthesis; whereas other *Hox* genes are regulated only indirectly by the same signal (the present work, Chapter 4). Subsequent *Hox* expression in the neuroectoderm depends on RA emanating from the non-organiser mesoderm (see Chapter 3 in the present work and references therein) but also FGF and Wnt signals (see General Introduction in the present work for references). It is thus obvious that different mechanisms are employed to configure the early A-P pattern, as determined by the *Hox* code, which can discern between the two tissues. However, it is conceivable that the characteristically nested expression of *Hox* genes in both germ layers arises from a more complex molecular network than the combination of three extracellular agents (Wnt, FGF and RA), even if those are modulated in time and space. On the other hand, given the coincidental collinear array of *Hox* expression domains in the two germ layers (Wacker et al., 2004; See General Introduction in the present

work), it is tempting to speculate on the existence of some kind of coordination between these tissues.

A possible mechanism, albeit unconventional, to account for *Hox* pattern coordination is communication via homeoproteins. *Hox* genes final products, homeoproteins, contain a domain characteristic of the superfamily they belong to. This homeodomain confers them DNA binding activity, by means of which they act as classical transcription factors. However, a long line of research work has uncovered and highlighted the potential of homeoproteins as secreted factors that are capable of nuclear export, secretion from and internalisation into cells *in vitro*.

In the present investigation we tried to examine the potential of homeoproteins to act as intercellular agents that carry information between the non-organiser mesoderm and the neuroectoderm. We speculated that, granted the particular physico-chemical properties of homeoproteins *in vitro*, in combination with the *in vivo* abilities some of them have to auto-regulate transcription of their own locus and to cross-regulate and modulate the transcription of other *Hox* genes, transfer of information by homeoproteins provides a potential mechanism to coordinate *Hox* pattern within the two tissues *in vivo*.

We tested this hypothesis by localised microinjection of mRNAs coding for the full-length HOXB4 homeoprotein, together or in the absence of a lineage tracer. We used histology and a combination of immunohistochemistry and in situ hybridisation techniques to follow spreading of the homeoprotein "signal" from a source (non-organiser mesoderm) to a recipient (neuroectoderm) tissue.

## Materials and Methods

Constructs and lineage tracers for microinjection

mHoxb4 mHoxb4 full length (1296bp) in T7TS, Hoxb4-myc Hoxb4 full length (aa 8-232) 5'myc CS2 (R. Morgan) and full-length GFP in CS2+.

Fluorescein dextran (MW 10,000) (Molecular Probes)

In situ hybridisation on paraffin sections of *Xenopus laevis* embryos

This protocol starts with sections which have been deparaffinised and rehydrated (see *Embedding and sectioning of* Xenopus laevis *embryos and explants in paraffin* for details). (See appendix A for a detailed protocol description).

Immunohistochemistry on sections of *Xenopus laevis* embryos and explants

Embryos and explants were harvested, fixed in MEMPFA and stored in absolute methanol; eventually, they were embedded, sectioned, deparaffinised and rehydrated as described in *Embedding and sectioning of* Xenopus laevis *embryos and explants in paraffin*. Rehydrated sections were washed four times in TBS, a couple of minutes each wash. The slides were then placed horizontally (sections facing up) into an incubation chamber (a plastic box, where sawed plastic tubes were fitted as holders, provided with a matching lid or parafilm and a wet tissue to keep a moist environment); a thick stripe was marked with a liquid repellent pen (liquid blocker super pap pen mini, Daido Sangyo Co.) beyond the uppermost sections, close to the holding end of each slide; immediately, the sections were carefully flooded with 400-500 µl of blocking solution (TBS, 0.1% Tween-20, 5% heat-inactivated lamb serum, 1 mg/ml BSA). After 25 minutes this solution was replaced with fresh blocking solution containing the primary antibody, and

incubated for at least 1 hour at room temperature (or overnight at 4°C). The antibody solution was removed and kept for reuse (thiomersal (Roche) was regularly added as a concentration of 0.01% to prevent infection and consequently antibody degradation) and the slides were washed at least four times 5 minutes in TBS. The slides were then placed back into the chamber and incubated in the secondary antibody, made in blocking solution, for at least one hour at room temperature. After this time, the slides were washed at least four times 5 minutes in TBS. For chromogenic reaction by secondary antibody-conjugated alkaline phosphatase, the slides were placed back into the chamber and rinsed twice with EKB. The alkaline phosphatase substrate (BM Purple (Roche) or FastRed (SIGMA*FAST* Fast Red (SIGMA)) was then added onto the sections and the wet chamber was placed in the dark.

Chromogenic staining took between 30 minutes and overnight to become a strong signal. The reaction could then be stopped by fixation, and sections were mounted and imaged as described in *In situ hybridisation on paraffin sections of* Xenopus laevis *embryos*. For immunostaining with fluorophore-conjugated secondary antibodies, the same procedure was followed for fixation, mounting (with DAPCO) and imaging of immunofluorescence, directly after the secondary antibody washes in TBS.

Antibodies used, dilutions and pairing

| *antigen* | *1ary Ab* | *2ary Ab* |
|---|---|---|
| GFP | polyclonal rabbit α-GFP IgG (1:400) (Invitrogen) | polyclonal goat α-rabbit IgG AP-conjugated Fab fragments (1:500) (SIGMA) |
| Hoxb4-myc | mouse monoclonal α-myc IgG (1:150) (9E10, Invitrogen) | polyclonal rabbit α-mouseIgG AP-conjugated Fab fragments (1:500) (Boehringer Mannheim) |
| Hoxb4-myc | mouse monoclonal α-myc IgG (1:150) (9E10, Invitrogen) | polyclonal goat α-mouse IgG Cy3-conjugated Fab frg. (1:200) |
| Fluorescein | polyclonal α-Fluorescein AP-conjugated Fab fragments (1:100) (Roche) | |

For double immunostaining, one of the antigens was detected following exactly the procedure described above. After development of the signal with either BM Purple or FastRed as a substrate, sections were fixed in MEMPFA for 1 hour (which should serve both to fix the colorimetric precipitate and to further inactivate the enzymatic reaction), then washed at least four times 5 minutes in TBS, and again incubated in blocking solution. The procedure above was repeated for the other antigen of choice, and the substrate for alkaline phosphatase was alternated, according to the one used in the first place. Once the chromogenic reaction corresponding to the second immunodetection had been developed (now two colours should appear), the sections were fixed and mounted as described above.

## Results

### Conservation of the SecPen in Hox genes of Xenopus laevis

A synthetic stretch of 26 amino acids (SecPen) encompassing the sequences essential for nuclear export and secretion on the one hand, and internalisation on the other, has been shown to be sufficient for translocation from cell to cell in mammalian cell cultures (Dupont et al., 2007). We wanted to assess whether HOX proteins also have the intrinsic potential to perform all these steps. In order to do that, we aligned the amino acidic sequences of several HOX proteins of *Xenopus laevis* with the *Drosophila melanogaster* Antennapedia sequence.

Analysis of a multi-alignment including Antennapedia and 11 Hox genes belonging to all four clusters and sampling members of 8 different paralogous groups, reveals a high degree of conservation in the homeodomain. In particular, the region spanning the putative Penetratin peptide, sufficient to drive internalisation and nuclear localisation into mammalian cells in culture (Derossi et al., 1994), presents a great homology among all the homeoproteins analysed, including Antannapedia (Figure 5.1A). 11 of the 16 aminoacidic positions aligning to the Antennapedia Penetratin sequence are strictly invariable in all the samples. Importantly, the WF doublet in positions 48-49 of the Antennapedia homeodomain, which is essential for cell internalisation of the homeoprotein (Derossi et al., 1994), is conserved in all cases. These results indicate that the ability to enter cells in a specific Penetratin-like fashion is intact in every *Xenopus laevis* HOX protein examined.

We wanted to investigate further the potential of HOX proteins to fulfil our hypothesis, that's to act as intercellular agents for communication between tissues. For that purpose, homeoproteins must not only cross the membranes of the receiving cells, but they also have to exit the nucleus and be secreted out of the emitting cells. This part of the process is known to depend on a stretch of 11 amino acids next to and partly overlapping with the Penetratin sequence in the third helix of the homeoprotein (Joliot et al., 1998; Maizel et al., 1999).

Multi-alignment of the same HOX proteins reported here above with Antennapedia and Engrailed2, in which requirement of the 11 amino acid stretch for secretion has been previously demonstrated (Maizel et al., 1999), shows a significantly lower degree of conservation among all the samples (2 of the 11 positions invariable), as compared to the Penetratin sequence (Figure 5.1B). However, two observations moderate these results. First, co-alignment with an additional homeoprotein (Emx2), which is expressed in the cerebellar cortex and olfactory neurons, where the protein is localised in the axons as well as in the nuclei (suggesting at least nuclear export, if not secretion) (Nédélec et al., 2004), reveals that 6 of the non-fully conserved amino acid positions also vary between En2 and Emx2; remarkably, 5 of these 6 positions are conserved among at least two of the HOX/Antennapedia proteins and either En2 or Emx2. Secondly, all of the 3 remaining non-fully conserved positions are only different in 1 of the homeoproteins. Altogether, these results, albeit showing a significant flexibility in the 11 amino acid sequence necessary for nuclear export and

secretion, don't argue against the possibility of a conserved secretory mechanism among homeoproteins.

In a parallel multi-alignment analysis, we focused on the HOXB4 homeoprotein to see the pattern of conservation within orthologues. Comparison of the homeodomains belonging to several species representative of a wide evolutionary range, revealed an absolute conservation of the Penetratin sequence in all samples (16 of 16 positions) and a significant, albeit lower, degree of homology (8 of 11 identical positions) in the 11 amino acid secretion sequence. The 3 non-fully conserved positions in the secretion sequence presented a relatively high degree of variability, even when compared to surrounding regions within the homeodomain. Remarkably, these 3 positions correspond to three also highly variable spots in the intraspecific HOX alignment described here above. These latter observations could indicate an important flexibility in some of the positions within the 11 amino acids stretch responsible for the secretory part of the intercellular transport of homeoproteins.

***In vivo* transcellular mobilisation of mHOXB4**

As a preliminary test for the intercellular capabilities of HOX proteins *in vivo*, we microinjected a full-length mRNA corresponding to the murine *Hoxb4* gene into a dorsal macromere of an 8-cells stage *Xenopus laevis* embryo. Targeting the mRNA into this particular blastomere was expected to lead to its distribution in the endoderm and dorso-lateral mesoderm of the older embryo. We incubated the embryos until stage 13, when mesoderm and neuroectoderm have been in close apposition during the whole process of gastrulation and remain separated by a thin interstitial gap (Brachet's cleft),

and then harvested them for immunohistochemical analysis on paraffin sections with an anti-mouse HOXB4 monoclonal antibody (Gould et al., 1998). This antibody has been raised against an epitope of the murine HOXB4 protein and has been reported to be quite specific, presenting no cross-reactivity with the chick orthologous protein. We could confirm this in our non-injected control embryos, where no significant staining was observed (Figure 5.2A). In the same analytical conditions, embryos injected with the mouse *Hoxb4* mRNA (*mHoxb4*) showed a distinct chromogenic reaction. Localisation of the ectopic HOXB4 was seen largely in the endoderm and dorsal-lateral mesoderm in most embryos; however, in some cases and in a low proportion of cells this distribution included other tissues like the neuroectoderm. Interestingly, close observation presented focal points of the signal where localisation in both mesoderm and neuroectoderm formed a contiguous domain. That was more evident in tailbud embryos (stage 21), when the gap between the two tissues is better seen (Figure 5.2B). Even though it is not unthinkable that cells on both sides of the gap (i.e. belonging to the either tissue) originate from the same injected blastomere and segregate into different tissues to finally come to lie by each other after gastrulation, the graded nature of the chromogenic signal encourages the idea that HOXB4 can spread between cells and eventually tissues.

**Distribution of ectopic *Xenopus laevis* HOXB4 as compared to a lineage tracer**

Two different reasons led us to adopt an alternative strategy to ectopic expression of a mouse HOXB4. On the one hand, our observation that the

anti-mouse HOXB4 is not cross-reactive with the endogenous HOXB4 in *Xenopus laevis* was challenged by a parallel observations. When *Xenopus laevis Hoxb4* mRNA (*XlHoxb4)*, rather than *mHoxb4* mRNA, was ectopically expressed in an embryo, there was a significant increase in the "background". Upon analysis by whole-mount immunohistochemistry, this background was often reminiscent of the endogenous *Hoxb4* transcript distribution (as assessed by in whole-mount in situ hybridisation (see Figure 3.3 and 3.4 for examples)) (5.3A) and accumulated distinctly on top of a more generalised background also seen in non-injected control embryos (Figure 5.3B). The same observation was made upon exposure of non-injected embryos to RA (Figure 5.3C), which is known to upregulate endogenous *Hoxb4* expression (Lumsden and Krumlauf, 1996; Durston et al., 1998; Godsave et al., 1998). Bearing in mind the documented positive feedback of HOXB4 proteins onto their own locus (Gould et al., 1997), these new observations suggested the possibility that in both manipulative conditions high non-physiological levels of endogenous HOXB4 protein may result in cross-reactivity by the anti-mouse HOXB4 antibody. On the other hand, co-injection of a lineage tracer with the *Hoxb4* mRNA was required to reveal the cells originally targeted by the microinjection and eventually discern them from the spreading domain of the HOXB4 protein. Because the intensity of the signal yielded by detection with the former antibody was not satisfactory, double immunostaining was bound to be suboptimal.

To circumvent these problems, we then decided to overexpress a *Hoxb4* mRNA containing a single myc tag in its 5' end (*XlHoxb4-myc* (in collaboration; R. Morgan). Attachment of the tag permits a clean distinction

between endogenous and ectopic HOXB4. Importantly, the position of the tag in the amino-terminal end of the protein is presumably not likely to interfere with endogenous functions, as the most active domain (homeodomain) is situated in the opposite carboxi-terminal end (Furukubo-Tokunaga et al., 1993 and references therein). Following the same targeting approach as above, *XlHoxb4-myc* was co-injected with a fluorescein dextran for traceability of targeted cells. In parallel, and in order to enhance the reliability of our results, we used an *in vitro* assay which consisted of dissecting a piece of non-organiser mesoderm extracted from a donor embryo that had been co-injected in the two blastomeres at the 2-cells stage with *XlHoxb4-myc* and fluorescein dextran, and placing it between two animal caps from non-injected control embryos. This should allow to finely discern the fully-loaded non-organiser mesoderm from the naïve neuroectodermal tissue (a piece of organiser mesoderm dissected from a non-injected control embryo was added to emulate endogenous neural induction events (Wacker et al., 2004)). Double immunostaining with anti-myc and anti-fluorescein antibodies on paraffin sections of stage 13 embryos showed no obvious distribution of HOXB4-myc protein outside the injected (fluorescein labelled) domain. However, the distribution of fluorescein in the tissue sections was obviously much more extensive than that of HOXB4-myc (Figure 5.4A). Remarkably, in our *in vitro* assay, even when focal points of mesoderm cells labelled with anti-myc antibody (always co-labelled by anti-fluorescein antibody) were seen in apposition to the naïve neuroectoderm, no obvious spreading of the signal into the latter tissue was seen (Figure 5.4 B). We suspected that the lineage tracer could be

interfering with the stability of either the *XlHoxb4-myc* mRNA or the HOXB4-myc protein, a consideration supported by the fact that some co-injected embryos showed extensive distribution of fluorescein and seldom or no detection of HOXB4-myc (data not shown). Moreover, experiments in which a piece of non-organiser mesoderm from an embryo co-injected two times at the two cells-stage with *XlHoxb4-myc* and fluorescein dextran was dissected and grafted into the marginal zone of a host gastrula embryo, presented a fluorescein labelled well integrated piece of tissue, which however showed very reduced levels of HOXB4-myc (data not shown).

**Distribution of HOXB4-myc to GFP proteins**

Assuming the possibility that the dextran-based fluorescein might be interfering with our experiments by enhancing mRNA degradation, either prior to microinjection or within the cytoplasm itself, we then went on to use another mRNA as a lineage tracer. A very good antibody allowed clean and strong distinction of GFP injected cells, and the same happened with individual detection of myc with the anti-myc antibody used here above. Unfortunately, double immunostaining with these two antibodies led to very poor signal of the second detection step (regardless of the sequential order). However, we thought that an alternative strategy could also be valid, albeit not optimal, where a paraffin ribbon that contains sections following an order in the length that corresponds to their relative position in the depth of the sample was divided in fragments of two sections. Every other fragment was placed on either a slide to be immunostained with the anti-GFP antibody or a slide to be immunostained with the anti-myc antibody; in that way, alternate, two-by-two consecutive sections could be analysed and the

overlapping/non-overlapping distribution domains between GFP and HOXB4-myc could be assessed. Analysis following this strategy led to observation of focal points where ectopic HOX protein was seen often in solution of continuity between two apposed domains of non-organiser mesoderm and neuroectoderm, respectively. Interestingly, the pattern of these domains is graded and seems to diffuse from one tissue to another across the interstitial gap, where the signal is often intensely detected (Figure 5.5B). Moreover, other domains that do not localise within the vicinity of the cleft (e.g. deep into the mesoderm or even endoderm) also show a graded pattern emanating from an intense core of signal (Figure 5.5B). As opposed to that, detection of GFP protein in consecutive sections show a much sharper domain boundary, in some cases corresponding to the cleft that appears otherwise to be crossed by the HOX protein (Figure 5.5A).

**Distribution of HOXB4-myc protein to *XlHoxb4-myc* mRNA**

We thought that detection of the ectopic *XlHoxb4-myc* mRNA could be used as a very reliable lineage tracer. We used the same strategy as above for GFP and HOXB4-myc, except that in this case consecutive sections were processed for immunostaining with the myc antibody on the one hand and in situ hybridisation on the other hand. Despite the fact that we used a probe that recognises both ectopic and endogenous *XlHoxb4* transcripts, we could detect spreading of HOXB4-myc protein into the basal side of the neuroectoderm epithelium (Figure 5.6B) outside the reference of the mRNA domain (contained within rather sharp boundaries, as was the case with the GFP protein) (Figure 5.6A).

**HOXB4 translation efficiency**

Regardless of the ultimate observations that suggest a possible spreading of the HOX signal across cellular membranes and interstitial spaces, our results provide a limited and still weak evidence to support this hypothesis. One of the reasons could lie on the fact that a relatively low percentage of the cells targeted by microinjection express detectable levels of HOXB4-myc protein, as estimated from reference of GFP protein distribution. Moreover, the fact that the domain of HOXB4-myc protein, albeit largely coincidental with its mRNA expression, is very scattered as compared to the mRNA suggests a restriction at the level of efficiency of translation. Because the ectodermal layer of the early *Xenopus laevis* embryo contains considerably lower amounts of yolk than the more internal layers (especially the endoderm), it is reasonable to think that it could provide a better scenario for HOX protein diffusion. Additionally, because tissues with lower lipidic concentration tend to show lower intensity of autofluorescence, we thought that immunofluorescence combined with co-addition of a fluorescent lineage tracer could provide better resolution for our analysis. To directly visualise potential spreading of HOX protein and assess at the same time the efficiency of its translation from an mRNA, we microinjected whole embryos with either fluorescein dextran or *Hoxb4-myc* mRNA; animal caps from the two types of microinjected embryos were dissected and combined heterotopically one-to-one, to be cultured until the non-operated embryos reached approximately stage 16. Visualisation of paraffin sections from these samples after immunodetection of HOXB4-myc protein with a TRITC-conjugated secondary antibody, revealed a striking complementarity

between the signals ensuing from fluorescein labelled and myc-labelled cells, respectively (Figure 5.7). Remarkably, whereas the fluorescein signal formed a cohesive and continuous domain the complementary area presented a scattered display of labelled cells (Figure 5.7).

**Discussion**

Here, we have tested the *in vivo* ability of homeoproteins to act as signalling factors that can spread across cell membranes. We believed that HOX proteins in particular may behave as specific messengers that convey the nested pattern displayed by *Hox* genes in the *Xenopus laevis* gastrula, from the non-organiser mesoderm onto the closely apposed neuroectoderm.

Multi-alignment of the homeodomain of up to 11 *Xenopus laevis* HOX proteins and the *Drosophila melanogaster* HOX orthologue Antennapedia, shows a strong conservation of the aminoacidic region essential for *in vitro* internalisation of homeoproteins into cells. A parallel multi-alignment of these same sequences in addition to two extra homeoproteins shows a much poorer conservation of the aminoacidic region essential for nuclear export and secretion from cells *in vitro*. This latter unexpected result can be attenuated by comparison between the sequences of the two extra added homeoproteins. There is evidence that supports the ability of both these proteins, En2 and Emx2, to exit the nucleus and/or cell *in vivo*. Despite this fact, En2 and Emx2 present a relatively low homology (5/11 positions conserved) between them. Hence, suggesting a remarkable flexibility in the aminoacidic region essential for nuclear export and cell secretion. In support of this view, a multi-alignment of HOX4 orthologues from several species

encompassing a large evolutionary range reveals a strict conservation (16/16 positions) in the internalisation sequence and a significantly more relaxed sequence for the nuclear export and cell secretion (8/11 positions conserved) region. We conclude that *Xenopus laevis* HOX proteins may retain the potential to act as intercellular agents, albeit a striking flexibility in the aminoacidic stretch that grants the nuclear export and cell secretion abilities. It is therefore worthwile to experimentally test the whole mechanism in the embryonic context of interest.

Ectopic expression of *Hoxb4* full-length mRNA, followed by immunohistochemistry against the overexpressed protein, shows graded localisation of the signal from a strong staining centre (hypothetically the nucleus of a cell targeted with the mRNA) to a distal and faintly stained periphery that appears to take the immediately neighbouring cells. Co-injection of *GFP* mRNA as a lineage tracer, followed by alternate immunolocalisation of HOXB4 and GFP proteins in consecutive sections of the same sample, presents distribution of the HOX protein outside the domain of overlap with the GFP protein. The same picture is obtained when distribution of the *Hoxb4* mRNA itself is detected by in situ hybridisation and used as a reference. Importantly, in both experimental settings localisation of the HOXB4 protein shows a continuous and yet irregular distribution over an area covering at least a couple of cells, suggesting that this domain neither is constrained to the nuclei of targeted cells nor it seems to obey to the topography of cell boundaries. Furthermore, the fact that the ectopic HOX protein is in some samples localised in the Brachet's cleft (extracellular gap separating the closely apposed non-organiser mesoderm and

neuroectoderm) and across this natural boundary in a solution of continuity over the two neighbouring tissues, strongly suggests that ectopic HOXB4 can diffuse out of and probably also into cells in the embryonic context of interest. In the same instances (i.e. consecutive sections), either the GFP protein or the *Hoxb4* mRNA domains sharply localise within the boundaries of the mesoderm and don't invade the Brachet's cleft.

Although these results seem to show that a HOX protein can diffuse across nuclear and cell membranes in a specific manner (unlike the lineage tracers) in a biologically relevant situation (even the one we are interested in), they don't yet provide strong evidence to support our testing hypothesis. The latter relies on a HOXB4 (or other HOX proteins) positive auto-regulatory feedback loop to enable the transfer of regional *Hox* identity from the non-organiser mesoderm (NOM) to the overlying neuroectoderm. This implies that a bunch of HOXB4 proteins (or eventually other HOX proteins) ensuing from the NOM cells should be able to cross over the Brachet's cleft and into the neuroectoderm cells and their nuclei. Once the HOXB4 proteins have reached these nuclei, a self-sufficient *Hoxb4* (*Hox* specific) induction event could start in the neuroectoderm that should establish the *Hoxb4* expression domain in this tissue, regardless of a continuous HOX protein input from the NOM. Nevertheless, results presented in this work show a significant and yet limited spreading of the HOX proteins, which rarely seem to go beyond the basal side of the neuroectoderm (proximal to the NOM and the Brachet's cleft) and are therefore unlikely to eventually reach the nuclei of these cells. Hence, provided the magnitude of such a spreading, we were thus far unable to obtain solid support for our hypothetical mechanism.

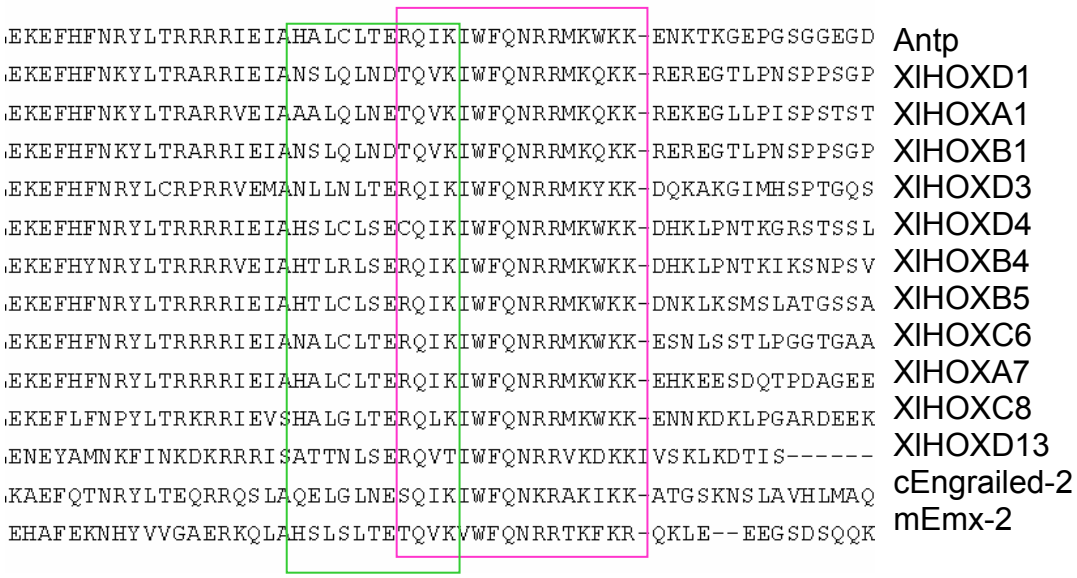
On the other hand, our observations also seem to indicate a relatively poor production of the exogenous HOXB4 protein, both after co-injection of *mHoxb4* together with a lineage tracer or upon *mHoxb4* injections alone, which renders a scattered localisation of HOXB4 protein in the non-organiser mesoderm, spreading from intense but focal centres that presumably correspond to nuclei of some NOM cells only. Comparing this pattern to the hypothetical (as assessed from endogenous *mRNA* distribution) steady and cohesive endogenous HOXB4 production arising from virtually every cell in the NOM throughout gastrulation, an explanation for the "insufficient" limited spreading of exogenous HOXB4 under our experimental conditions could be accounted for. Moreover, because we cannot detect the levels and distribution of endogenous protein, which is expected to provide a continuous renewal of protein via the auto-regulatory feedback loop, we are underestimating the consequences of our exogenous application, as this could be inforcing the production of endogenous HOXB4 and thus mimicking the hypothetical physiological situation.

Altogether, we can conclude that our results provide evidence that suggests HOX proteins may act as transcellular agents in vivo during gastrulation. Nevertheless, whether such a mechanism naturally accounts for or participates in the establishment of the *Hox* pattern profile in the embryo requires further proof. It seems that the unability thus far to give stronger and further support to our starting hypothesis is hindered by techcnical aspects rather than contradicting results. Namely, the apparent little robustness of protein production in our experimental assay fails to emulate fully the presumed situation in the embryo proper. We predict that

optimalisation of the expression construct in order to improve the HOX protein yield should reveal the viability of this mechanism. Furthermore, experiments where endogenous HOXB4 protein is depleted in the mesoderm by means of targeted microinjected of a specific morpholino against the pertaining mRNA, complemented with targeted co-injection of exogenous HOXB4 protein from the expression construct, should give definitive proof.

***Figure 5.1.*** **Multi-alignment of homeoproteins shows evolutionary conservation of the Pen and Sec sequences.** (A) homeodomain alignment of sequences corresponding to 11 HOX proteins, Antennapedia, chicken Engrailed-2 and mouse Emx-2. (B) homeodomain alignment of sequences corresponding to HOXB4 protein homologues from different vertebrate species or the HOX4 orthologues from a few invertebrate species. The 16 amino acids spanning the Penetratin sequence in Antennapedia and their counter-aligned positions in the other homeodomains are framed within a pink box. The 11 amino acids spanning the Sec sequence in Engrailed-2 and their counter-aligned positions in the other homeodomains are framed within a green box. The name of each homeoprotein is written on the left side of the panel.

***Figure 5.1***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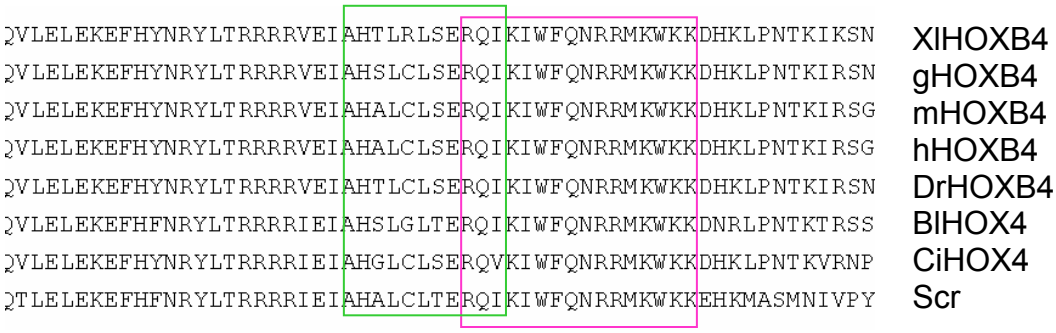
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QVLELEKEFHYNRYLTRRRRVEIAHALCLSERQIKIWFQNRRMKWKKDHKLPNTKIRSG mHOXB4
QVLELEKEFHYNRYLTRRRRVEIAHALCLSERQIKIWFQNRRMKWKKDHKLPNTKIRSG hHOXB4
QVLELEKEFHYNRYLTRRRRVEIAHTLCLSERQIKIWFQNRRMKWKKDHKLPNTKIRSN DrHOXB4
QVLELEKEFHFNRYLTRRRRIEIAHSLGLTERQIKIWFQNRRMKWKKDNRLPNTKTRSS BlHOX4
QVLELEKEFHYNRYLTRRRRIEIAHGLCLSERQVKIWFQNRRMKWKKDHKLPNTKVRNP CiHOX4
QTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEHKMASMNIVPY Scr

**Figure 5.2. Tissue localisation of mHOXB4 protein targeted to mesoderm.** Transverse sections along the A-P axis of stage 21 *Xenopus laevis* (A) non-injected control embryos and (B) embryos microinjected with mouse *Hoxb4* mRNA. Both samples were processed for immunohistochemistry with an anti-mouse HOXB4 antibody. Staining is visible as a dark blue precipitate.

***Figure 5.2***

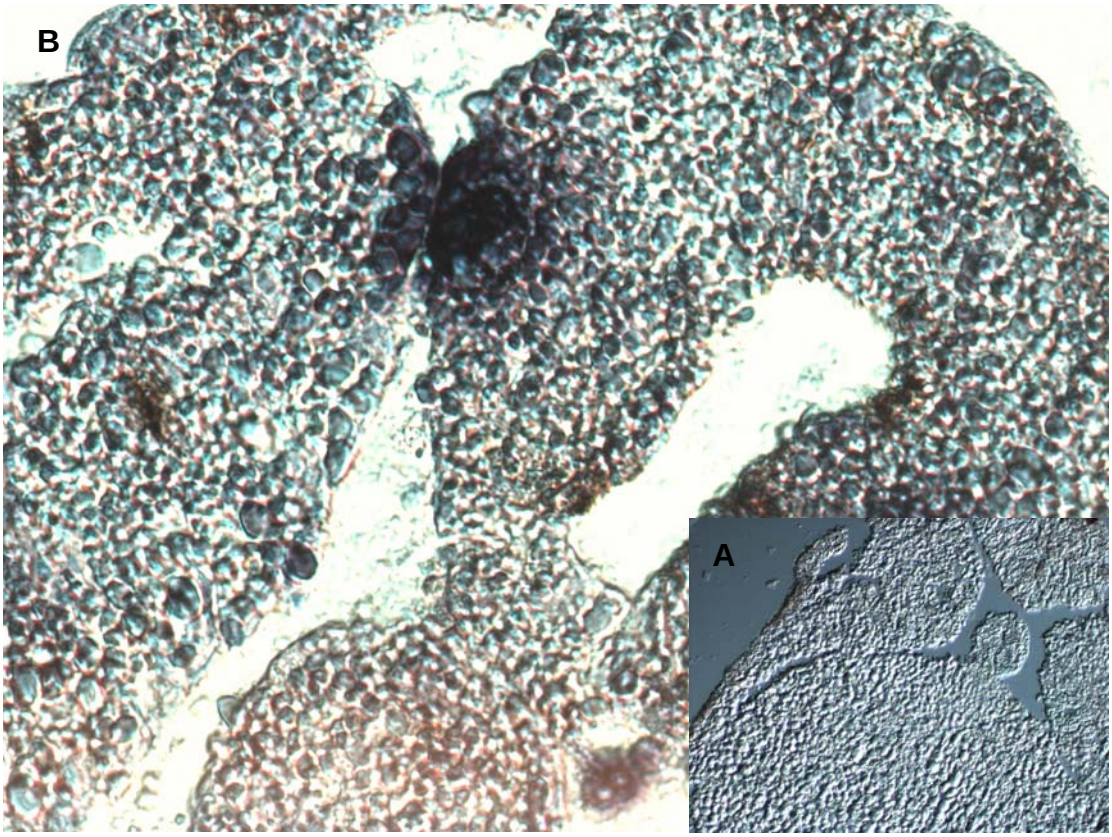


***Figure 5.3.*** **The anti-mouse HOXB4 antibody cross-reacts with XlHOXB4 protein in embryos analysed by whole-mount immunohistochemistry** (A) after overexpression of *XlHoxb4* mRNA or (C) upon exposure to RA, whereas (B) non-injected non-treated control embryos present only a slight background. Lateral views of tailbud embryos.

***Figure 5.3***

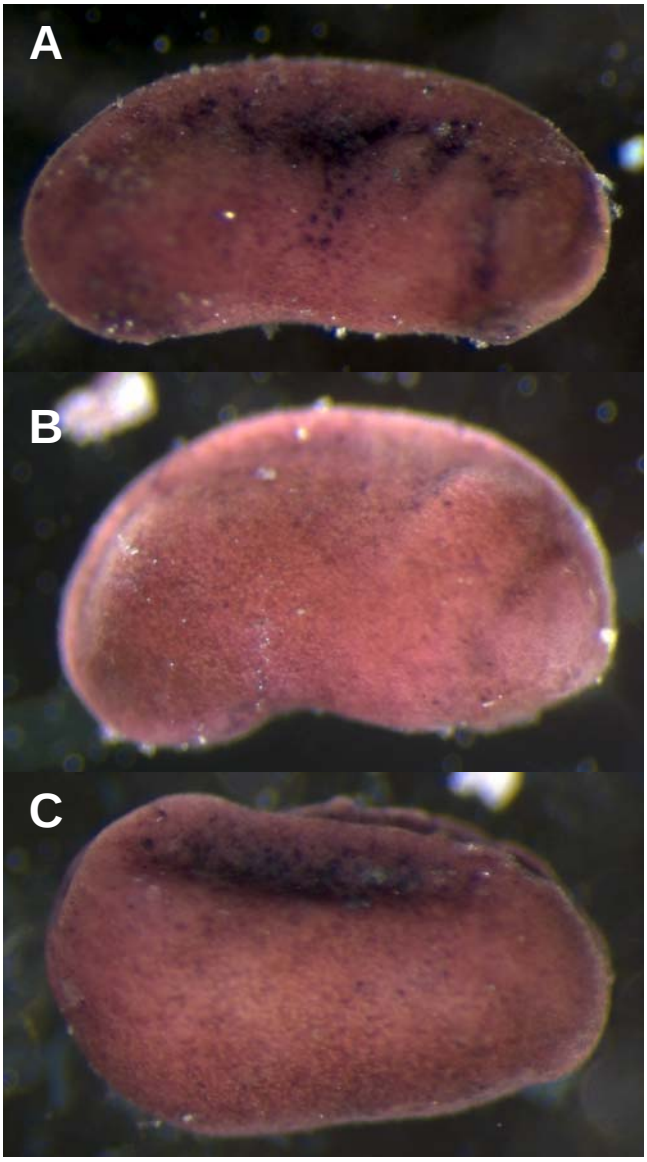


**Figure 5.4. Co-detection of Fluorescein dextran and XlHOXB4**

Double immunohistochemistry on paraffin sections (A) along the A-P axis of stage 13 embryos after co-injection of Fluorescein dextran and *XlHoxb4-myc* mRNA or (B) explant recombinates where the non-organiser mesoderm had been loaded with Fluorescein dextran and *XlHoxb4-myc* mRNA. The red precipitate corresponds to Fluorescein localisation and the dark blue precipitate to HOXB4-myc.

***Figure 5.4***

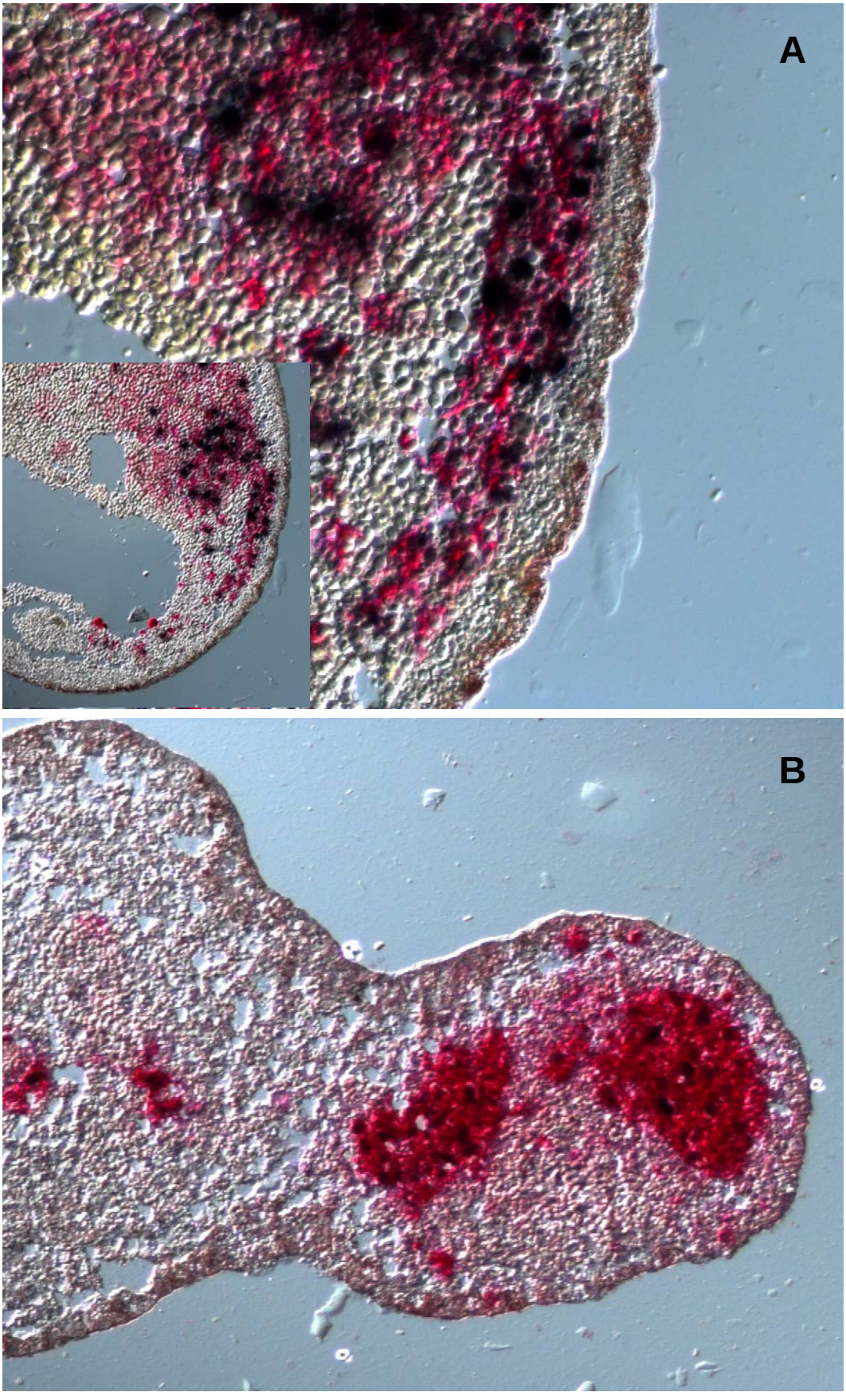


***Figure 5.5.*** **Detection on consecutive sections of GFP and XlHOXB4.** Immunohistochemistry on consecutive paraffin sections along the A-P axis of one single embryo, after co-injection with *GFP* and *XlHoxb4-myc* mRNAs, shows large overlap in the domains localising both ensuing protein types, but also graded diffusion across the Brachet's cleft of HOXB4-myc proteins (B) but not GFP (A). Each panel displays increasing magnification views of one section, as 10x, 20x and 40x from top to bottom. The rectangular frames correspond to the region enlarged in the subsequent lower image.

***Figure 5.5***

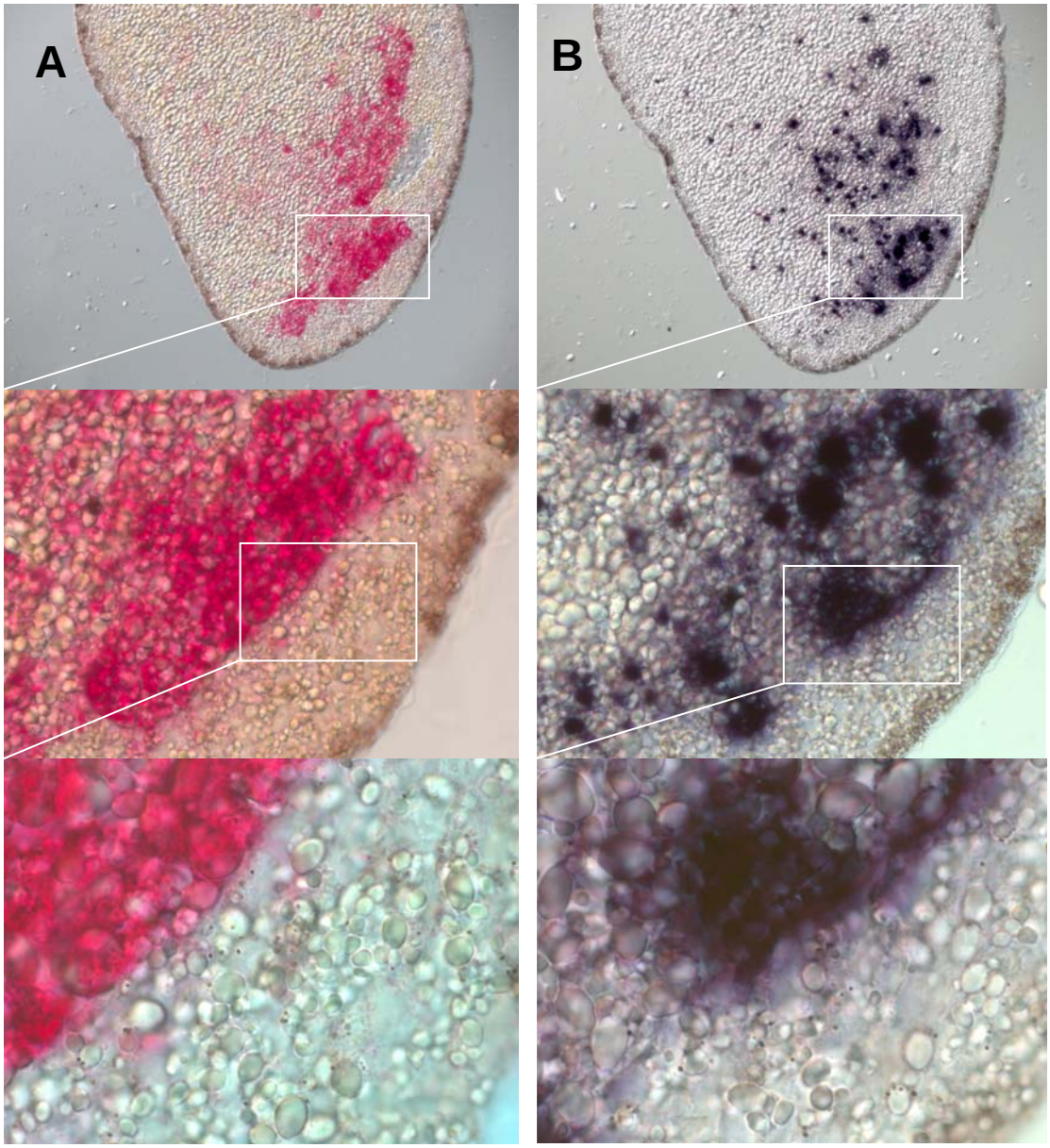


***Figure legend 5.6*. ISH and immunohistochemistry on consecutive paraffin sections** along the A-P axis of the same embryo, after targeted microinjection of *XlHoxb4-myc* mRNAs, shows large overlap in the domains localising the mRNA and the protein, but also graded diffusion across the Brachet's cleft of the protein (B) but not the mRNA (A). Each panel displays increasing magnification views of the same section, as 10x, 20x and 100x from top to bottom. The rectangular frames correspond to the region enlarged in the subsequent lower image

***Figure 5.6***

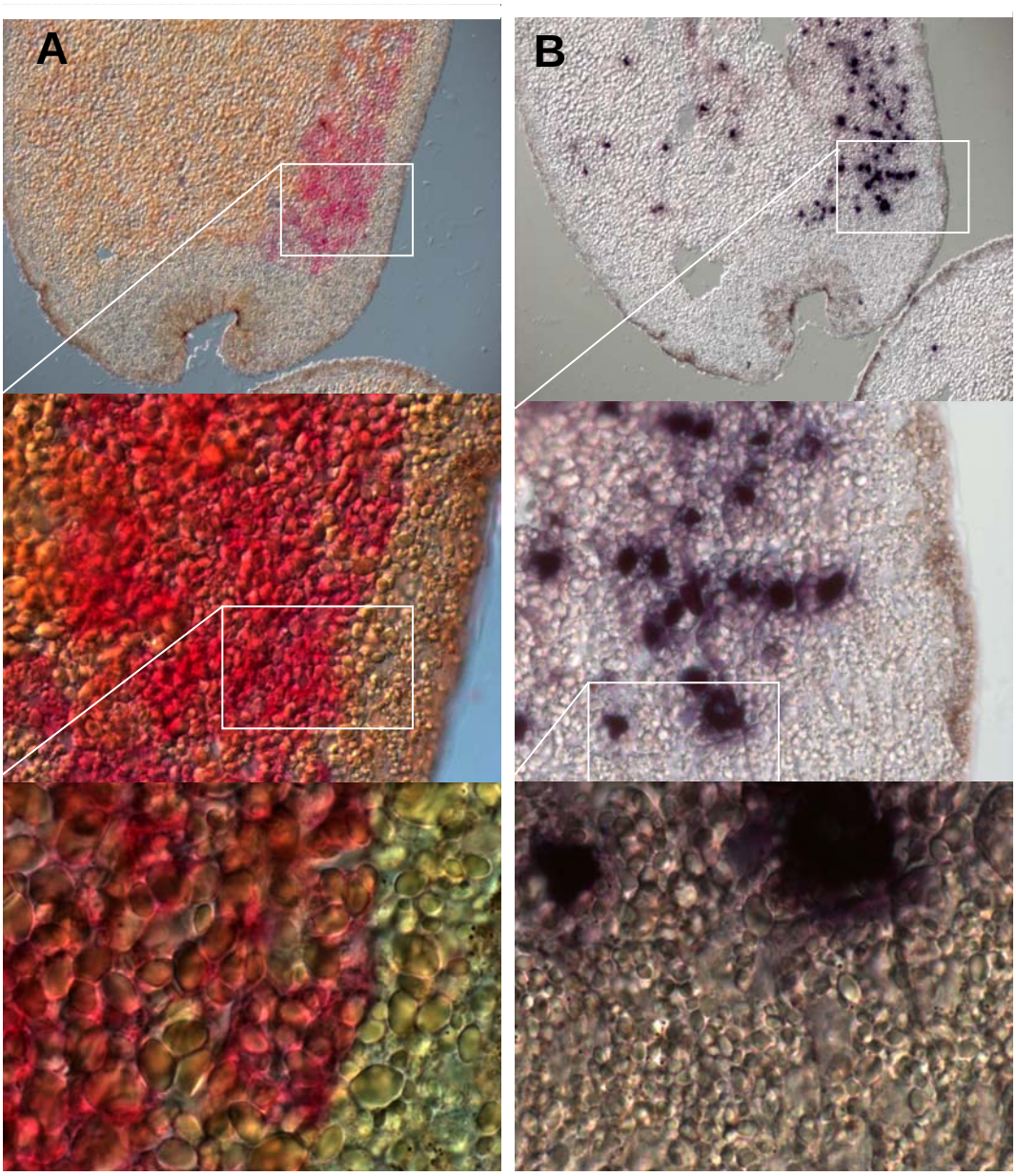
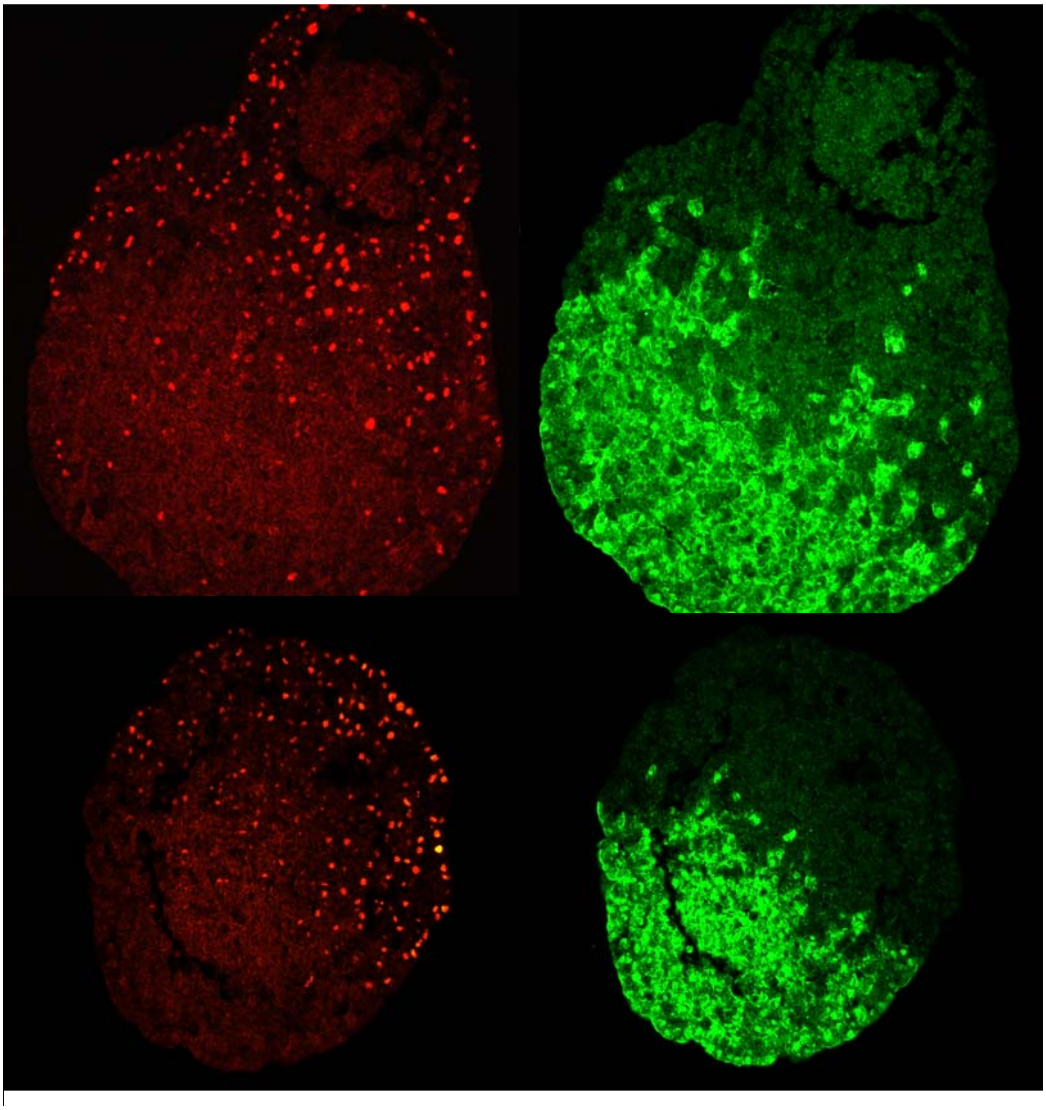


***Figure 5.7.*** **Immunofluorescence on consecutive paraffin sections of recombinates made of animal caps** dissected from embryos microinjected with Fluorescein dextran only and animal caps from embryos injected with *XlHoxb4-myc* only. Consecutive sections of the same recombinate are arranged from left to right and two different samples are shown in the top and the bottom images, respectively. Green fluorescence localises Fluorescein dextran, whereas red fluorescence localises HOXB4-myc.

***Figure 5.7***



**Chapter 6**

## General Discussion

In the present work I have been investigating the molecular mechanisms that initiate and establish a correct pattern of *Hox* expression domains in the early vertebrate embryo. In the first place, the timing and source of retinoid signalling required for the earliest *Hox* patterning of the neural tissue has been directly identified. In *Xenopus laevis* retinoid activity is found to be necessary during gastrulation to induce 3' *Hox* genes in the anterior neural plate, but expression of the same genes in the adjacent non-organiser/paraxial mesoderm is not dependent on this activity. Moreover, the non-organiser mesoderm is shown directly to be the source of the retinoid signal that imparts the *Hox* pattern in the neuroectoderm. Furthermore, evidence is given for the requirement of *Wnt* signalling in the earliest phase of *Hox* expression during gastrulation, which takes place in the non-organiser mesoderm and is retinoid-independent. This requirement includes all *Hox* genes analysed (both 3' and 5'). Moreover, the Wnt pathway is shown to act directly, that is in the absence of protein synthesis, to induce labial (*Hox* 1 p.g.) members, but indirectly on the expression of other *Hox* genes. Importantly, the effects seen after attenuation of *xWnt8* activity are milder at the end of gastrulation, at least regarding the expression of some *Hox* genes. By this time, the initial phase of expression in the non-organiser mesoderm has been shifted to a more intense domain in the neuroectoderm, suggesting different tissue sensitivity.

Altogether, these results indicate that patterning along the anterior-to-posterior (A-P) axis of the non-organiser mesoderm on the one hand and of the neural tissue on the other, involve different mechanisms and signalling molecules. It is evident from the data in the present work that retinoids are only instructive in the anterior neural tissue. Regarding Wnt signalling activity however, data presented here doesn't allow to sharply distinguish between effects on the two tissues. However, the fact that effects of *Xwnt8* knock down on *Hox* expression at the early gastrula are attenuated by the early neurula, suggests that neuroectodermal *Hox* expression either is not Wnt-dependent or it is the result of a synergy between Wnt and other signals. Regardless of that, the milder effects seen at early neurula could arise from an indirect repercussion of the early *Hox* downregulation in the mesoderm (except for *Hoxc6*, which is upregulated; see Discussion in Chapter 4). Although that would necessarily imply a connection (and perhaps coordination) between the *Hox* patterns in the two tissues (discussed later in this chapter). The relatively mild *Hox* downregulation observed upon XWNT-8 depletion at the early neurula, coinciding with the peak of neuroectodermal expression, raises the question whether the morphological phenotype evoked in the tadpole embryo may be a result of early *Hox* alterations. Specific loss-of-function of *Xwnt8* in the present work and general blocking of Wnt signalling in previous approaches (see Introduction in Chapter 4 for references), shows a strong anteriorisation of the axis, including a considerable upregulation of cement gland markers and a concomitant downregulation of *Krox-20* (McGrew et al., 1997), the latter result indicating that anteriorisation of the axis includes neural tissue.

Interestingly, McGrew et al show no effect on *Hoxb9* expression levels at stage 23 (although in Keller explants), a gene which is strongly expressed in the neural tube (Curran and Grainger, 2000; and own observations). Thus, whereas Wnt activity is necessary for correct *Hox* expression in the early gastrula non-organiser mesoderm, there is the possibility that the morphological phenotype of Wnt loss-of-function revealed at later stages in the neural tube either indirectly reflects alterations in the mesoderm (perhaps related to the early gastrula *Hox* downregulation) or it is caused by Wnt actions on phenomena other than *Hox* regulation. This could be related to the head inducing capabilities of the Wnt inhibitor genes *Dickkopf-1* and *Cerberus,* both of which are secreted from the head mesendoderm (Glinka et al., 1998; Piccolo et al., 1999). The possibility of an interference with the isthmic organiser activity is less likely, because our work especifically targets *Xwnt8* function, and only *Wnt1* seems to be expressed in this region (see General Introduction). Finally, even though the direct induction assay in the present work (Chapter 4) shows strong upregulation of some *Hox* genes in the neuroectoderm, as well as in the mesoderm, it is a gain-of-function approach and therefore doesn't necessarily reveal endogenous situations. Co-factors of the Wnt transduction pathway required for *Hox* expression and present in the gastrula non-organiser could be missing in the neuroectoderm. Alternatively, differential states of competence between both tissues could also account for selective sensitivity to Wnt signalling.

**Retinoids and Wnt, graded morphogens or simple activators**

It seems that Wnt and retinoid signalling act to trigger initial expression of *Hox* genes in mesoderm and neuroectoderm, respectively. *Hox* genes are

known for their expression patterns, particularly in the early vertebrate embryo, which result in an array of nested domains along the A-P axis that follows their position in the chromosome (reviewed in Fienberg et al., 1987).These restricted expression domains determine identities of and correspond to structures (segments) in the embryo (reviewed in Fienberg et al., 1987). Hence, precise establishment of these domains is crucial in determining the body plan. Retinoids and Wnts, next to FGFs, are the main signalling factors associated with laying down the early *Hox* pattern (the present work; see General Introduction and Chapters 3&4 for references) and therefore they are expected to instruct their target tissues as morphogens, i.e. by eliciting a range of differential responses on cells distributed across or along a tissue or tissues (Papageorgiou, 1980a,b). Such mode of tissue characterisation (i.e. pattern) by a signalling molecule implies generation of a gradient, which is spread across the receiving tissue (Papageorgiou et al., 1980a,b). Going back to the specific situation in the gastrula embryo, the morphogen principle should be translated in: two signals Wnts and retinoids, forming two respective gradients; non-organiser mesoderm and neuroectoderm, as two receiving tissues; and *Hox* genes, as the responding factors, whose differential expression (pattern) is the origin of tissue characterisation.

A big amount of data relating to studies on A-P patterning of the nervous system has been interpreted as a confirmation of the existence of a retinoid gradient in the mid-axial neural region (i.e. posterior hindbrain and anterior spinal cord), with *Raldh2* in the trunk paraxial mesoderm providing the source of signal and *Cyp26* RA-degrading enzymes as the inhibitors that

generate the sink (Chen et al., 2001; Reintjes et al., 2004; Maves and Kimmel, 2005; White et al., 2007; Strate et al., 2009). However, other studies, including direct measurement of RA along the A-P axis (Maden et al., 1998), suggest a non-instructive role of retinoid signalling and homogeneous levels of RA across the posterior hindbrain region (Maden et al., 1998; Gale et al., 1999; Hernández et al., 2007).

Regarding a proposed Wnt gradient involved in patterning the A-P axis (Kiecker and Niehrs, 2001), results in the present work do not conform very well to this idea (discussed in Chapter 4). However, I cannot exclude existence of a Wnt morphogen providing regionalisation along the A-P axis in a way that doesn't imply *Hox* genes, or at least not early on. Work in Thomas Edlund's group indicates an instructing role for a posterior-to-anterior increasing gradient of Wnt signalling, which encompasses and acts upon the whole length of the A-P axis (Nördstrom et al., 2002; Nördstrom et al., 2006). Nonetheless, the latter studies reveal that the Wnt dose-dependent specification of the neural tissue (albeit in an explant *in vitro* system) exerts a rough pre-compartmentalisation of the A-P axis. In fact, in the most recent of their studies (Nördstrom et al., 2006), they show that specification of the posterior hindbrain and anterior spinal cord occurs relatively late in the Nieuwkoop transformation process, when fore- mid- and anterior hindbrain but also posterior spinal spinal cord are already specified, and that it requires RA to act upon the pre-specified anterior regions and induce posterior hindbrain genes. Additionally, Wnt and other inhibitors emanating from the head mesendoderm/anterior visceral mesoderm seem to determine distinction between fore- and midbrain (Glinka et al., 1998;

Piccolo et al., 1999; Hashimoto et al., 2000). Besides, during neurulation *FGF8* and *Wnt1* conspicuous expression in the MHB isthmic organiser are essential for determining midbrain and anterior hindbrain identities (see General Introduction). Interestingly, work in Thomas Edlund's group itself using the same *in vitro* system, show that explants isolated from late gastrula epiblast already possess intrinsic potential to generate an isthmic organiser, and that pre-determination of this inducing region is dependent on FGF and Wnt (the same combination required along the entire A-P axis, but not sufficient to generate all regions in the axis) (Olander et al., 2006).

It is thus not clear whether morphogen gradients are active in the early embryo to pattern the A-P axis. However, I argue that the evidence presented so far can be explained by both with or without the existence of such gradients, at least regarding retinoids and Wnts. If one considers establishment of the A-P patterning, particularly in the well-studied neural tissue, how is this regional specification achieved in case of absence of morphogens? One plausible, and yet unusual explanation would involve a set of specific determinants, each having the intrinsic potential to trigger differentiation of and hence grant identity to a particular region. *Hox* genes comply very well with this characteristic. But how is their differential expression attained in the neural tissue in the first place? For in the last instance, a non-pre-formistic view of development of the embryo demands a step or more steps that cause heterogeneity and eventually morphogenesis. One doesn't need to go so far to explain the case of neural tissue patterning, if the instruction arises from the adjacent non-organiser/paraxial mesoderm (and still assuming lack of a retinoid or Wnt morphogens).

In the present work (Chapter 5), I have been investigating the possibility that HOX proteins themselves, each one of them carrying the information to characterise a precise region of the neural tissue along the A-P axis, can directly export their differentiating capabilities from the already patterned non-organiser/paraxial mesoderm to the neural plate. The *in vitro* well-characterised properties of homeoproteins (e.g. HOX proteins) have not extensively been tested *in vivo*. Here I examined the potential of HOX proteins, and particularly HOXB4, to act as intercellular messengers. That involves nuclear export and secretion from the *Hoxb4* transcript expressing cells in the early gastrula non-organiser mesoderm (Wacker et al., 2004; the present work), and uptake from the extracellular space by the neighbouring neouroectodermal cells. I find that the ectopic *Hoxb4* mRNA targeted to the former tissue produces HOXB4 protein that eventually localises in the adjacent (initially non-targeted) cells of the neuroectoderm and accumulates across the interstitial gap (Brachet's cleft) that separates the two tissues. My results are still preliminary, among other aspects because spreading of the HOXB4 protein is not detected far from its original source (i.e. less than one cell layer) in my observations (Chapter 5). In order to support the role of HOX proteins as intercellular messengers to account for a possible mechanism of pattern coordination between non-organiser mesoderm and neuroectoderm, the homeoproteins should display a longer range of action. Specifically, this hypothetical mechanism relies on HOX proteins eventually reaching the nucleus of the receiving cells in the neuroectoderm. Once there, thanks to the self-regulatory properties known for some HOX proteins (including HOXB4) (Gould et al., 1997), the activation and further

propagation of the message may be triggered. The new site of *Hox* transcription in the first line (proximal to the Brachet's cleft and non-organiser mesoderm) of neuroectodermal cells might provide an endogenous source of HOX protein in the neuroectoderm that progressively and autonomously spreads across the tissue. This gradual self-propagation could be sufficient to explain part of the dynamic expression patterns of *Hox* genes in the gastrula of *Xenopus laevis* embryos, with initial appearance in the non-organiser mesoderm and subsequent activation in the overlying neuroectoderm (Wacker et al., 2004).

I conclude, after a series of observations resulting from different experiments (Chapter 5), that the lack of stronger evidence to favour a potential role of homeoproteins as intercellular messengers may be due to the excessively low levels/scattered distribution of expression, and in particular translation, yielded in my experimental design. If one compares these to the endogenous domain of *Hox* expression (at least mRNA levels) in the gastrula non-organiser mesoderm, it is reasonable to think that the assays utilised in the present work cannot emulate a presumptive physiological situation, particularly because they fail to provide sufficient HOX signal intensity and homogeneity.

Finally, assuming that the aforementioned mechanism is active in the embryo and that retinoids don't establish a morphogenetic gradient, it is possible that the two signals still interact. In fact, the strong induction power of RA on and specially its requirement for 3' *Hox* genes expression (see General Introduction for references and present work) suggests that this signal is crucial, if not as an instructor, as a co-factor of the presumptively

signalling HOX proteins. In this scenario, it is tempting to bring in RA and its capabilities as an activator through RAREs present in some *Hox* genes' promoters (see General Introduction for references), to synergise and potentiate the auto-regulatory loop of *Hox* genes.

Finally, regarding the question of how the very initial *Hox* pattern in the gastrula non-organiser mesoderm is set up,,much less is currently known Identification in the present work of Wnt signal requirement for the earliest *Hox* expression phase, and particularly its potential as direct inducer of labial *Hox* genes, is a first step in the elucidation of the mechanisms involved.

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# Appendix A: protocols

## Whole-mount in situ hybridisation of *Xenopus laevis* embryos

- fix at least 4 hrs with 50 µl MEMPFA buffer per embryo

  (optional: overnight at 4°C)
- replace MEMPFA with absolute MeOH
- wash 1x for 15 min with absolute MeOH
- store at -20°C

*Hybridisation*

- rehydrate embryos with 5-minute washes in absolute MeOH, 75% MeOH-25% water, 50% MeOH-50% water, 25% MeOH-75% TBS-Tween , and 3x in TBS-Tween
- incubate embryos in 2ml of approx. 150 ng/ml proteinase K (optimised per new batch of proteinase K), made up in TBS-Tween, for 25 min at 50°C in a shaking water bath
- rinse the embryos for 5 min in 1 ml 0.1 M triethanolamine, pH 7-8 (TEA)
- rinse the embryos for 5 min in 1 ml TEA with 2.5 µl/ml acetic anhydride (mix solutions well before use)
- after 5 min add 0.5 ml TEA with 10 µl acetic anhydride and mix gently
- rock for 5 min (Do not incubate longer!)
- wash the embryos twice for 5 min in TBS-Tween
- refix for 20 min in MEMPFA
- wash 5x 5 min in TBS-Tween
- transfer embryos to small glass vials
- add 500 µl hybridisation buffer (PreHyb)

- rock for 10 min at room temperature
- replace solution with 500 μl hybridisation buffer
- prehybridise for 4-6 hr at 65°C
- add 10-200 ng/ml probe
- hybridise overnight at 65°C

*Washing*

- remove and store the hybridisation buffer with the probe for reuse and add 500 μl hybridisation buffer
- add 500 μl 2x SSC and shake at 65°C for 20 min
- remove 500 μl of the mix and add another 500 μl 2x SSC
- shake at 65 °C for 20 min in a water bath
- wash the embryos 3x for 20 min with 2x SSC at 65°C (all wash volumes are 1ml)
- incubate the embryos in 2x SSC containing 5 μg/ml Rnase A at 37°C for 30 minutes
- wash three times in 0.2x SSC for 20 min at 65°C
- wash the embryos twice in MNT for 10 min at room temperature

*Antibody incubation*

- remove the MNT and replace with 500μl blocking buffer
- rock for at least 1 hr at room temperature
- replace the solution with a fresh solution of blocking buffer containing a 1:3000 dilution of anti-digoxigenin antibody Fab fragment coupled to alkaline phosphatase (Boehringer-Mannheim) (500μl per 20 embryos)
- rock for 4 hr at room temperature

- to remove excess antibody wash the embryos 3x 5 min and 2x 10 min at room temperature with MNT
- wash at least 5 hours and preferably also overnight with MNT at room temperature
- wash 2x 10 min in TBST
- transfer the embryos to clean vials
- for the chromogenic reaction with alkaline phosphatase, wash the embryos twice, 5 min each at room temperature, with alkaline phosphatase buffer
- replace the last wash with the same solution containing 3.5 µl of NBT and 3.5 µl of BCIP per ml of buffer or with BM-purple solution. Use 0.5 ml / 20 embryos, and incubate at room temperature. Keep the reaction in dark and start with rocking for 30 min, then keep vials upright in closed dark box.

The colour reaction is visible starting after approx. 20 min to 1 day. Stop the chromogenic reaction when satisfied with signal and background by replacing the solution with MEMPFA. The staining procedure lasts between 20 minutes and three days. One can temporary stop the staining reaction storing the vials on ice-water. The staining can be speeded up by placing the vials at 30°C.

- after at least 4 hr, wash 3x 10 min, then 2x 1hr and subsequently overnight in absolute MeOH
- if necessary store at -20°C

**In situ hybridisation on paraffin sections of *Xenopus laevis* embryos**

*Pretreatments*

- incubate slides two times 5' in TBS
- wash two times in 5' TBS/100mM glycine
- incubate 15' TBS/0.3% Triton X-100
- rinse two times in 5' TBS
- 30' 37°C Prot.K in TE (100mM Tris 7.6, 50mM EDTA, 10μg/ml Proteinase K)
- incubate for 5' with prechilled (4°C) 4% paraformaldehyde in TBS (or MEMPFA)
- wash two times 5' with TBS
- incubate the slides in TEA 0.1M pH 8.0/0.25% acetic anhydride for 5' followed by a 5' incubation in TEA 0.1M pH 8.0/0.5% acetic anhydride

The protocol can be interrupted at this point; if desired, follow the next two steps.

- dehydrate in 50-70-90-100-100% EtOH
- rinse briefly with chloroform

The slides can now be stored dry in a clean box at -20°C.

*Hybridisation*

- put slides in a humid chamber at 55°C 30'
- add 200-400μl hybridisation buffer and incubate at 60-70°C for at least 1 hour
- replace the hybridisation buffer with hybridisation buffer that contains 5-500 ng/ml probe and hybridise o/n

*Post-hybridisation washes*

- wash twice in 2*SSC for 30’
- incubate 2*SSC containing 5 µg/ml RNAse A for 30’ at 37°C
- wash three times 20’ with 50% formamide/1*SSC at 55°C (*important*: this step is crucial to be able to go back to solutions with lower concentration of salt, without significant loss of sections; meanwhile, it retains the high-stringency required to help washing away the remaining unspecific binding of probe)
- wash two times 15’ in 1*SSC at room temperature
- rinse three times 5’ with TBS

*Antibody incubation and chromogenic development*

- incubate 15’ in TBS that includes 1% BB
- incubate for 60’ in TBS/1% BB/$\alpha$-DIG-AP 1:2000 at room temperature
- wash the glass slides thoroughly in TBS (at least 2 hours, but a longer washing procedure results in less a better signal-noise ratio)
- incubate the slides10’ in EKB
- detect with NBT/BCIP (3.5µl NBT and 3.5µl BCIP per ml EKB) or with BM-Purple
- the staining has to be performed in the dark and can take 10’ to 2 days

Fix the sections in MEMPFA for at least 15 minutes, when satisfied with the staining; then wash a few times in TBS. For mounting and imaging see *Embedding and sectioning of* Xenopus laevis *embryos and explants in paraffin*.

**Whole-mount in situ hybridisation of chick and quail embryos**

*Pretreatments*

- Rehydrate embryos through 75%, 50% and 25% methanol in PTW (PTW=calcium-magnesium free PBS with 0.1% Tween-20), allowing embryos to settle between changes
- Wash 2x with PTW, 10 min each
- For embryos older than about 2 days, bleach for 1 hour in 6% H2O2 (1ml H2O2 + 4ml PTW from 30% stock)
- Wash 3x with PTW, 10 min each. For the last wash, measure the volume of PTW (use 2 or 5 ml, depending on size of tube).
- Add Proteinase K (1:1000; final concentration = 10µg/ml). Incubate at room temperature for 30 min, regardless of stage of embryos, but reduce this to 15 min for New cultured embryos. During incubation, gently roll the tube every few minutes to make sure the sides and top of vial get wet with Proteinase K.
- Take off Proteinase K and rinse briefly with a very small volume of PTW (carefully!)
- Replace PTW with 4% formaldehyde in PTW (doesn't need to be fresh), containing 0.1% glutaraldehyde. Postfix 20-30 minutes.

*Prehybridisation and hybridisation*

- Remove postfixing solution, and wash 2x briefly with PTW
- Remove PTW, and replace with 1 ml hybridisation solution
- Remove hybridisation mix, and replace with another 1-2 ml (5 ml if using large vials)
- Place tube upright in a beaker in water bath at 70°C. Incubate 2-6 hours

- Remove hybridisation mix, and replace with probe in hybridisation mix

*Post-hybridisation washes*

- Remove probe and keep at -20°C for reuse
- Rinse 3x with a small volume (<1ml) prewarmed hybridisation solution
- Wash 2x with 1.5 ml (4 ml if large vial) prewarmed hybridisation solution, 30 min in water bath
- Wash 20 min with prewarmed 1:1 hybridisation solution : TBST

*Post-antibody washes*

- Remove antibody solution
- Rinse 3x with TBST
- Wash 3x 1 hour with TBST, rocking (fill vial right up to the top). (Older embryos need more washing).
- Block embryos with 5% heat inactivated (at 55°C for 30 min) sheep serum in TBST with 1 mg/ml BSA, 3 hours
- Remove blocking buffer from embryos and replace with fresh blocking buffer containing anti-DIG alkaline phosphatase conjugated antibody diluted 1:500
- Incubate overnight at 4°C on a rocking platform
- Wash 2x 10 min with NTMT
- Incubate in NTMT containing 4.5 µl NBT (75mg/ml in 70% DMF) and 3.5 µl BCIP (50mg/ml in 100% DMF) per 1.5 ml, rocking, protected from light, at room temperature. Alternatively, incubate in BM-Purple or other alkaline phospatase substrate.

After the signal has been developed, stop by washing 2x 10 minutes in TBST or PBS.

## Appendix B: solution recipes

### SSC 20X recipe

- Dissolve the following in 800ml of distilled H2O.
    - 175.3g of NaCl
    - 88.2g of sodium citrate
- Adjust the pH to 7.0 with a few drops of 1M HCl.
- Adjust the volume to 1L with additional distilled H2O.
- Sterilise by autoclaving.

### TBS 1X recipe

- Dissolve the following in 800ml of distilled H2O.
    - 8g of NaCl, 0.2g of KCl
    - 3g of Tris base
- Add 0.015g of phenol red.
- Adjust the pH to 7.4 with HCl.
- Add distilled H2O to 1L.
- Sterilise by autoclaving.

### PBS 1X recipe

- Dissolve the following in 800ml distilled H2O
    - 8g of NaCl
    - 0.2g of KCl
    - 1.44g of Na2HPO4
    - 0.24g of KH2PO4

- Adjust pH to 7.4
- Adjust volume to 1L with additional distilled H2O
- Sterilise by autoclaving

**PBST 1X recipe**

- Dissolve the following in 800 ml of distilled H2O
    - 8g of NaCl
    - 0.2g of KCl
    - 1.44g of Na2HPO4
    - 0.24g of KH2PO4
    - 2ml of tween-20
- Adjust pH to 7.2
- Adjust volume to 1L with additional distilled H2O
- Sterilise by autoclaving

**MEMPFA recipe**

- *M* MOPS (3-(N-morpholino)propanesulfonic acid), pH 7.4
- m*M* EGTA
- m*M* MgSO4
- 3.7% formaldehyde

A stock solution is prepared not containing the formaldehyde.

1 part of a 37% formaldehyde stock solution is added to 9 parts of buffer/salt mix just prior to use.