Hox in frogs: xenopus reveals novel functions for vertebrate Hox genes

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A toutes les petites lumières qui ont éclairé ce chemin long et tortueux : merci !

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Chapter 1

Introduction

Early Development and Axis Formation in *Xenopus Laevis*

Early dorsal-ventral axis determination by breaking symmetry.

Multicellular organisms are not symmetrical and possess well defined axes of polarity, the dorso-ventral (back-belly) and anterior posterior (head-tail) axes being most obvious. One of the major challenges in biology is to understand how this polarity is achieved during embryogenesis and how ordered structures are generated along the body axes of a developing embryo. For over a century, the amphibian embryo has played a major role in these investigations, mainly due to its large size and external fertilization, facilitating the analysis of the early steps in the development and patterning of the embryonic axes.

Initially, the still unfertilized amphibian egg (from for instance *Xenopus laevis*, South African clawed frog) is radial symmetrical. Upon fertilization, sperm entry (which can occur anywhere in the animal hemisphere) causes the outer layer (the cortex) to loosen from the dense yolky core cytoplasm (Fig.1) and initiates the process of cortical rotation. This process results in a ~30° displacement of the vegetal cortex away from the sperm entry site and towards the future dorsal region (reviewed by Gerhart *et al.*, 1989; Houliston and Elinson, 1992) and is associated with the translocation of a maternal dorsalizing activity from the vegetal pole towards the prospective dorsal side of the embryo (Kikkawa *et al*., 1996; Kageura, 1997). This displacement of maternal determinants leads to the stabilisation on the future dorsal side of the canonical Wnt pathway effector, β-catenin. This dorsal stabilisation of nuclear β-catenin is the first event that determines dorsal-ventral polarity in the *Xenopus* and zebrafish embryos (Hibi *et al.,* 2002).Later on during development, the region where β-catenin is stabilized will develop into the so called Nieuwkoop center (De Robertis *et al*., 2000; Gerhart, 2001; Weaver and Kimelman, 2004), a group of cells that is responsible for induction of the Spemann organizer (reviewed in De Robertis and Kuroda, 2004). Kuroda and colleagues have shown that β-catenin also induces the formation of a second signaling center in the dorsal ectodermal cells called the **B**lastula **C**hordin and **N**oggin **E**xpressing region (BCNE, Kuroda *et al*., 2004). The latter seems to be involved in and required for central nervous system formation (Fig.2, and Kuroda *et al*., 2004).

The processes mentioned above take place before gastrulation during blastula stages of the embryo. At the beginning of gastrulation the embryo had thus changed from a radial symmetrical to a polarized state which subsequently will allow the generation of both the dorso-ventral (DV) and anterio-posterior (A-P) axes.

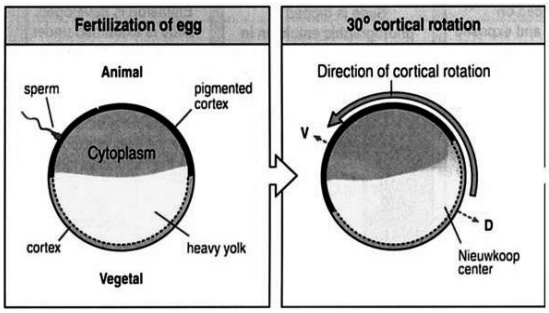

Figure 1: ***Xenopus* egg fertilization leads to cortical rotation.** The sperm entry at the animal pigmented pole triggers a loosening of the yolk from the cortex (left panel) which is followed by a cortical rotation. This results in displacement of maternal components from the vegetal pole towards the future dorsal side of the embryo. Thus fertilization breaks the egg symmetry and specifies the ventral-dorsal (and indirectly the anterior-posterior) axis of the embryo (scheme from Wolpert, Principles of development)

From cells to an organized embryo.

In what is usually referred to as the most famous experiment in embryology, Hans Spemann and Hilde Mangold showed that a specific region in early amphibian embryos, the dorsal blastopore lip, can induce a second complete embryonic axis including the head, when transplanted to the ventral side of a host embryo (Spemann and Mangold, 1924)

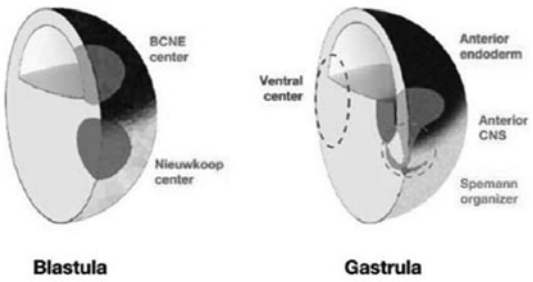

Figure 2: **Important early signaling centers within the early embryo.** Formation of the Nieuwkoop signaling center triggers the formation of the Blastula Chordin and Noggin Expressing region (BCNE) during the blastula stage. Mesoderm induction is accompanied by formation of the Spemann organizer dorsally. Cellular movements during gastrulation implicate part of the BCNE in formation of the presumptive anterior neural derivatives (scheme from De Robertis and Kuroda, 2004).

Most of the tissues along the axis, including the nervous system, are derived from the host in which the graft induced cells to form an axis. Because of its ability to "instruct" other tissue, this specific region was named the organizer. While the original experiments were performed in newts, half a century later *Xenopus laevis* became the preferred model

for analyzing the molecular and genetic mechanisms involved in the formation of the Spemann organizer and its capacity to induce a properly patterned axis (reviewed in Stern, 2001; Dawid, 2004).

In the meantime, related structures in other model organisms were shown to have identical organizer functions (Niehrs, 2004). Respectively, in
the mouse, the gastrula organizer (later on during development referred to as the node) (reviewed in Levine and Hemmati-Brivanlou, 2007; Waddington, 1936; 1937), in the zebrafish the embryonic shield (Luther, 1935; Oppenheimer, 1936) and in chicken Hensen's node (Waddington, 1932; 1933; Joubin and Stern, 2001), correspond to the amphibian Spemann's organizer region.

All these organizer centers have in common the ability to "instruct" adjacent cells to participate in the process of axis formation. The Spemann-Mangold organizer is thus of central importance for the establishment of the vertebrate body axes. In addition to this, the organizer is involved in the patterning of the germ layers during early embryogenesis. Briefly, the organizer's main functions can be separated into: dorsalizing ventral mesoderm, inducing neural ectoderm and directing gastrulation movements (reviewed in De Robertis and Kuroda, 2004; Harland and Gerhart, 1997).

As molecules mediating the organizer functions, secreted BMP antagonists like Noggin and Chordin have been identified. In *Xenopus* and zebrafish, missexpression of these antagonists leads to induction of secondary axes and the formation of ectopic head structures. In addition, the same molecules are able to dorsalise mesoderm and to induce neural ectoderm in cells that would have otherwise developed into epidermal ectoderm. As a good understanding of the roles of the organizer in axial induction and neural patterning is essential to appreciate the research presented in this thesis, these patterning functions will be discussed in more detail below (reviewed in De Robertis and Kuroda).

Patterning functions of the organizer: axis formation

As mentioned above the ectopic expression of BMP antagonists on the ventral side of the *Xenopus* embryo recapitulates the induction of a secondary axis as in the Spemann experiment. Unexpectedly, mutants of BMP antagonists in mouse and zebrafish show only a mild axial phenotype or head defects (De Robertis and Kuroda, 2004), questioning the role of BMP antagonists in axis formation in vertebrates. However, in *Xenopus*, simultaneous knockdown of three BMP antagonists (Follistatin, Chordin and Noggin) leads to a dramatic loss of dorsal structures while knocking down any single one leads to a very

mild effect as seen in other vertebrates (Khokha *et al.*, 2005). Redundancy of anti-BMP molecules may therefore explain the mild axial phenotype caused by the loss of function of BMP antagonists in other organisms. A partial rescue of axial structures in the triple knockdown by knocking down BMP4 reinforces the idea that there is a strong requirement for BMP4 in early axis formation (Khokha *et al*., 2005).

Patterning functions of the organizer: neural induction

Beside its role in axial induction, Spemann organizer appears to have major functions in germ layer patterning. As gastrulation proceeds, the organizer was believed to instruct naive ectoderm to convert to neural tissue. This prompted a search for neural inducers that eventually led to the identification of several molecules with the expected properties (De Robertis and Kuroda, 2004; Stern, 2005). Three secreted factors were first identified in *Xenopus*: Noggin, Chordin and Follistatin which are all BMP antagonists (for review, see Harland and Gerhart, 1997).

Surprisingly, early studies revealed that gastrula animal cap cells that would normally develop into epidermis have the tendency to adopt a neural fate when dissociated. The addition of BMP4 is able to reverse this process and promotes epidermis formation (Wilson and Hemmati-Brivanlou, 1995; Hawley *et al.*, 1995). Altogether, this led to the idea of a "default model" of neural induction where ectodermal cells will primarily adopt a neural fate. In the embryo, this default state is suppressed in the animal cap by presence of BMP signaling but in a selected population of cells close to the organizer the default state is restored by organizer secreted BMP antagonists like Noggin and Chordin (Weinstein and Hemmati-Brivanlou, 1997). One should to be mention that the requirement of the Spemann organizer for the induction of neural tissue was questioned by other studies in different model systems. Physical removal of the organizer in chick, frog, zebrafish or mouse does not abolish the formation of neural tissue (Smith and Schoenwolf, 1989; Shih and Fraser, 1996; Davidson and Keller, 1999). In addition a more recent study suggested that initiation of anterior neural ectoderm in *Xenopus* (and in other vertebrates) takes place before gastrulation, excluding the requirement of signals from the Spemann organizer (Kuroda *et al.*, 2004).

In chapter II of this thesis, we present data suggesting that the organizer, actually makes ectodermal cells competent to receive patterning signals from the non-organizer mesoderm and that it thus promotes the formation of a complete and stable AP pattern (Jansen *et al.*, 2007).

In addition to BMP and BMP antagonists, the FGF signaling and Wnt signaling pathways also appear as key players in neural induction (Hongo *et al.,* 1999; Sasai *et al.,* 1996; Launay *et al.,* 1996; Aubert et *al.,* 2002; Baker *et al*., 1999). Different studies revealed that the organizer also secretes molecules able to antagonize other important signaling pathways, Wnts and Nodals (Gould and Grainger, 1997; Sasai and De Robertis, 1997; Niehrs, 1999; De Robertis *et al.,* 2001).

As it has been suggested by Linker and Stern *in vivo*, showing the absence of neural induction by antagonizing BMPs and Wnts in presence of FGF, more players might be involved (Linker and Stern, 2004). Despite the efforts of embryologists in investigating possible mechanisms responsible for the ability of the organizer to induce the nervous system; according, there are still many questions to be answered. Nevertheless, the overall picture depicts the Spemann organizer as a major signaling center defining the formation (at least in part) of dorsal tissues such as the neurectoderm and thus plays an important role in patterning the dorso-ventral body axis.

However, other signaling centers seem also to be critical in properly patterning the anterior-posterior (A-P) body axis (see below).

Anterior-posterior body axis patterning: Hox genes in control.

During gastrulation, polarity in the embryo along the body AP axis is being established and a clear distinction between different regions in the developing embryo will appear. At the basis of this is an A-P pattern of genetic specification that develops in the very early embryo (reviewed in Stern *et al*., 2006). Different gradients (such as FGF, Wnt and retinoic acid) and their different combinatorial activities trigger the expression of different genes at different A-P positions in the different germ layers, thereby defining different positional identities.

Homeotic mutations in *Drosophila* (in which one part of the body is transformed into a structure normally found at another A-P location) shed light on another class of genes involved in the proper patterning of the A-P axis of the animal; the genes causing these mutant phenotypes belong to a group of homeodomain genes, the *Hox* genes (Lewis, 1978). The *Hox* genes encode a family of transcription factors that are widely spread through the animal kingdom and are conserved throughout evolution (De Robertis, 2008).

In most vertebrates, *Hox* genes are organized in four clusters named A-D, located on four different chromosomes. Each cluster contains up to 13 genes. Similarly numbered

genes (1-13) from different clusters share a homologous evolutionary origin and are referred to as "paralogs". In general these genes share large parts of their expression domain (Fig.3) and have partially redundant functions (Fig.3, reviewed in McGinnis and Krumlauf, 1992; Amores *et al*., 1998). It is widely accepted that the 4 *Hox* clusters arose from an ancestral *Hox* cluster during the 2 rounds of whole genome duplication that occurred in the vertebrate lineages. After these duplication events changes such as gene loss and divergence in function occurred during evolution resulting in the current cluster organization (Prince and Pickett, 2002; Deschamps, 2007).

Within each cluster, genes at the 3'end are being expressed earlier during development and more anteriorly than genes positioned near the 5'end, which are expressed later and more posteriorly in the embryo. Thus *Hox* genes start to be expressed sequentially from the 3' towards the 5' of the cluster, a peculiar behaviour named "temporal colinearity". Their spatial expression sequence within the developing embryo also follows their order in the cluster, and this is called "spatial colinearity"(Kmita and Duboule, 2003; Gaunt, 2000; Deschamps *et al*., 1999). This spatio-temporal colinearity leads to a unique combination of expressed HOX proteins at a specific A-P position, referred to as the "*Hox* code"(Duboule and Morata, 1994; Kessel, 1994).

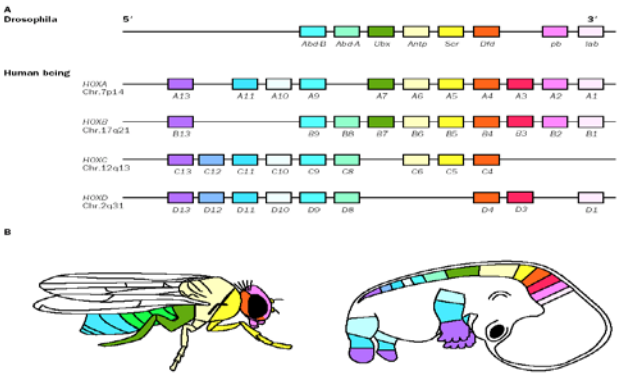

Figure 3: **Chromosomal organisation of Hox clusters and their expression pattern in Drosophila and a human embryo.** The fruit fly possesses a unique cluster while humans have four. Hox clusters are temporally and spatially colinear: genes located at the 3' end of the cluster and more rostrally expressed within the animal than genes located towards the 5' end. Similarly numbered and colored genes represent the same paralogous group (1-13). Hox genes have a colinear pattern also in the forming limbs (Goodman, 2003).

In the *Xenopus* embryo, *Hox* gene expression is initiated very early in a broad domain in the ventro-lateral mesoderm of the marginal zone, thus excluding the Spemann organizer domain. This initial domain corresponds to the area where the BMP4 and *Xbra*

domains of expression overlap (Wacker *et al*., 2004b). Here the Hox gene expression follows a temporal colinear sequence and an interaction with the Spemann organizer seems to then stabilise *Hox* gene expression; thus stabilizing *Hox* codes that otherwise would have only a transient existence as different cells bearing the same Hox signature are brought within the signaling range of the organizer different time points. Thus, involution and convergence-extension movements of the mesoderm during gastrulation lead to a stable spatial *Hox* expression, translating the temporal pattern into a spatial pattern as explained by "the time-space translation model"(Wacker *et al*., 2004a; reviewed in Stern *et al.*, 2006). In the mouse, the formation of *Hox* gene expression patterns along the A-P axis seems to be the result of several steps: *Hox* expression has to be initiated during gastrulation in the posterior primitive streak, in the "Hox induction field" or the so-called "*Hox* opening zone" (Gaunt, 2000; Deschamps *et al.*, 1999), followed by an establishment phase in which the *Hox* domain of expression spreads to generate the definitive pattern. Finally, this expression domains are stabilized during a third step, the maintenance phase (reviewed in Deschamps *et al*., 1999).

Current data suggest that in higher vertebrates, the initial epiblastic expression of *Hox* genes is conveyed to the mesoderm during gastrulation and subsequently spreads its pattern to the neurectoderm (reviewed in Deschamps *et al.*, 1999). In the frog, the initial expression of *Hox* genes is located in the mesoderm, and as in other vertebrates, this pattern subsequently spreads to the neurectoderm. In chapter VI of this thesis, we present preliminary data pinpointing towards a possible mechanism of *Hox* expression "spreading" into the neurectoderm.

In vertebrates, most *Hox* genes have a sharp anterior border of expression at well defined axial level, while their expression domains, overlap caudally, in a nested fashion. The increased number of HOX proteins expressed at a more posterior level does not correlate with the presence of more complex caudal structures. It has been actually shown that where multiple paralogous *Hox* genes are expressed at the same level, the most posterior genes are dominant versus more anterior ones, and determine the positional information, a phenomenon which is referred to as "posterior prevalence"(Duboule and Morata, 1994).

Genetic studies have shown that functional compensation can occur between paralogous *Hox* genes. Indeed, loss-of-function (LOF) of a single *Hox* gene in the mouse often leads to a very subtle phenotype, and even the removal of the complete HoxD cluster gives a very mild phenotype (where only the atlas was transformed into the axis (Zákány *et al.*, 2001)). An approach where all members of a paralogous group are inactivated can cause a more dramatic homeotic transformation where the region of expression of those

genes will adopt a more posterior phenotypic character. This for instance was shown by the inactivation of *Hox4* paralogues genes (Horan *et al.,* 1995). Similarly, we have shown that the knockdown of the paralogous group 1 in *Xenopus* leads to a more severe hindbrain phenotype in comparison to the LOF of each single *Hox1* gene alone (McNulty *et al.,* 2005, chapter IV of this thesis).

Hox gene regulation

Hox genes are the main genes involved in patterning the anterior-posterior axis. The upstream activating factors of these genes thus play a crucial role during early axis establishment. This section will not review all the data about *Hox* genes regulation. It will only further summarize recent data on *Hox* regulation; for more extensive insights, I'll refer to some very good recent reviews: Iimura and Pourquié, 2007; Svingen and Tonissen, 2006; Akin and Nazarali, 2005. One of the most studied activating factor of *Hox* genes is Retinoic Acid (RA). Its role in axis patterning was shown previously in *Xenopus,* zebrafish and mammalian embryos, suggesting a very conserved function in axial patterning. Treatment of early embryos with RA led to anterior truncations where anterior neural tissue adopts a more posterior fate as seen at tadpole stages (Durston *et al.,* 1989; Sive *et al.,* 1999). This effect affects both the mesoderm and neurectoderm layers (Durston *et al.,* 1989; Ruiz I Altaba and Jessell, 1991). RA was shown to be responsible for the cranial expansion of 3' *Hox* genes (Kolm *et al*., 1997; Godsave *et al*., 1998) thereby contributing to patterning the anterior-posterior axis. Thus, *Hox* gene expression in the hindbrain is thought to be established by RA partly (reviewed in Guthrie, 2007). RA ability to induce *Hox* gene expression was shown *in vitro* in a study, where a temporally colinear activation was triggered (Simeone *et al.,* 1990). *In vivo* studies showed that RA is needed somehow for the anterior spreading of anterior *HoxB* genes within the hindbrain (Oosterveen *et al*., 2003). Moreover, RA derived from the rostral somites is thought to specify the caudal hindbrain through the induction of *Hox* genes in a dose-dependent manner (Glover et al., 2006). A RA gradient seems to pattern hindbrain *Hox* gene expression (Sirbu *et al*., 2005; Godsave *et al*., 1998).

Similarly, FGF signaling is also able to modulate the expression of *Hox* members within the nervous system (Muhr *et al.,* 1999; Storey *et al.,* 1998). In the hindbrain, FGF8 secreted by the isthmus area (mid-hindbrain boundary) sets the anterior boundary of *Hoxa2* expression (Irving and Mason, 2000). Factors such as *Krox-20* (also called EGR2) and *kreisler* (named MAFB) activate the transcription of *Hox* genes which contain an *Antennapedia*-class homeobox.

Autoregulatory loops between different *Hox* members have been described in *Xenopus* (Hooiveld *et al*., 1999) and mouse (Tümpel *et al.,* 2007; Manocochie *et al.,* 1997) adding a layer of complexity and suggesting that caution should be taken in interpreting the phenotype of single morpholino- and knock out. Even though, *Hox* gene function in body axis patterning has been investigated extensively for decades, very little is known about their downstream targets, one of the obvious reasons being the aformentionned redundancy. A very short overview of up to date data will be presented in the next section.

Hox gene regulation and their potential targets.

Hox genes are one of the most studied genes during development and the number of publications dealing with this topic has steadily increased during the last twenty years (Woltering, 2007). However, despite extensive interest in *Hox* genes, their downstream targets remain rather obscure. The introduction of techniques such as chromatine immunoprecipitation (ChIP) and microarrays has created exciting possibilities for the identification of *Hox* targets. The difficulties encountered in past approaches to discover *Hox* targets were likely due to the fact that some degree of redundancy exists between *Hox* members. Furthermore, HOX proteins have a weak DNA binding affinity, and their specificity seems to rely on the cooperative binding with co-factors like Pbx or Meis. It has been reported that other factors cooperate with HOX proteins in regulating some target genes: *Hoxa10* cooperate with a member of the *FoxO* subfamily of the forkhead transcriptions factors in regulating the Insulin-like growth factor binding protein-1 (reviewed in Moens and Selleri, 2006; Laurent *et al*., 2008). A manuscript not in the line of this thesis characterizing *FoxO* members in *Xenopus* is currently in preparation, and will not be presented here.

Despite all these difficulties, researchers were able to discover some *Hox* targets (reviewed in Akin and Nazarali, 2005; Svingen and Tonissen, 2006). The first target described in literature is the mouse neural cell adhesion molecule (N-CAM) (Jones *et al*., 1992). Some genetic studies have reported that factors like Gata3 are downstream targets of *Hox* genes in the developing hindbrain (Pata *et al.,* 1999). A collaborative approach in investigating the potential downstream targets of *Antennapedia* (*Antp*) in *Drosophila* and its *Xenopus* homolog *Hoxc6* is currently ongoing in Durston and Gehring's labs: we have ectopically expressed *Hoxc6* in *Xenopus* and *Antp* in Drosophila and monitored potential downstream targets by microarray experiments. The results will not be included in this thesis.

From neural tissue to neurons.

In a section above we already discussed the organizer and its role in the induction of the neural tissue. The initial neural tissue is however not patterned yet and does not show the differentiation typical for a functional nervous system. In all vertebrates, the morphological appearance of the embryonic nervous system takes place during neurulation: this process converts the so-called neural plate (flat tissue) into a neural tube and (Placzek and Furley., 1996). By the end of neurulation, a neural tube is formed, and different functional neuronal populations are present within this structure.

In the neural tube, different types of neurons are present at different positions along the anterior posterior axis. One of the fundamental issues in the developmental neurosciences is to understand the mechanisms that control the identity of these different classes of neurons located at different positions within the nervous system. The generation of the appropriate neuronal type at the appropriate position is crucial to establish the correct neuronal connections. Most of these characteristics are coordinately regulated and acquired during early development (for review, see Edlund and Jessell 1999; Jessell, 2000; Shirasaki and Pfaff, 2002).

Within the hindbrain as well as in the spinal cord, neurons are clustered in functional groups (Gilbert, 2006); motor neurons are organized in clutsers or nuclei in the hindbrain, while they form functional columns within the spinal cord (for review see Guthrie, 2007; 2004). *Hox* genes have been shown to be involved in patterning the hindbrain nuclei (reviewed in Matise and Lance-Jones, 1996; Briscoe and Wilkinson, 2004). Hox3 paralogues for examples were suggested to be implicated in the formation of somatic motor neurons (MNs) of the abducens and hypoglossal nuclei (Guidato *et al*., 2003; Gaufo *et al*., 2003).

Along the spinal cord, neurons are positioned in a columnar organization. This is linked to a the correct formation of axons projections and neuronal connections (Jessell, 2000). Classically, 3 columns are distinguished: medial motor column (MMC) neurons which innervate the body wall muscles; lateral motor column (LMC) neurons innervating the limbs; column of Terni (CT) neurons which project to the sympathic ganglia. Basically, MNs projecting to the same target belong to the same MN pool within the spinal cord (Jessell, 2000; di Sanguinetto *et al*., 2008). Several lines of evidence suggest that *Hox* genes contribute to the establishment of the pool identity of MNs within the spinal cord (Dasen et al., 2003; 2005). Hox proteins from Hox-C and Hox-D genes have been shown to be expressed by subsets of LMC neurons (Tiret *et al.*, 1998; Lance-Jones *et al.*, 2001). The columnar organization has been demonstrated to be under the control of sequential phases

of different Hox-C protein activities in response to some gradient molecules, including fibroblast growth factors. At the forelimbs level for instance, *Hoxc6* is playing a crucial role in defining the identity of MNs, while at the lumbar level, *Hox10* paralogues are more important (Dasen *et al.*, 2003; Bel-Vialar *et al.*, 2002; Liu *et al.*, 2001). Furthermore, *Hox* mutants have been shown to exhibit defects in axonal projections, consistent with an alteration in MN pools identity (Wahba *et al.*, 2001; Wu *et al*., 2008). For instance, targeted disruptions of *Hoxd9* and *Hoxd10* for instance cause alterations in peripherical nerve projections from specific motor pools (Carpenter *et al.*, 1997; de la Cruz *et al*., 1999).

Furthermore, in *Hoxc8* deficient mice, the topographic organization of motor pools that innervate forelimb distal muscles becomes disorganized (Tiret *et al.*, 1998). These findings support the idea that the action of the "Hox code" within MNs is a part of the mechanism that imposes on MNs the patterning information required for the fine tuning of the subtype identity necessary to establish connections between motor pools and muscles targets (Nordström *et al.*, 2006; Song and Pfaff, 2005).

In chapter III of this thesis, we will present data supporting a new role of *Hoxc6* gene in neurogenesis in *Xenopus*.

Axis elongation and its segmentation.

The segmented or metameric aspect of the anterior-posterior body axis is a basic characteristic of many animal species ranging from invertebrates to humans, and segmentation has long been thought to be a key aspect of the basic design of animals (Tautz, 2004). In vertebrates, segmentation addresses both the mesodermal tissue (the preseomitic mesoderm, PSM), and neurectoderm (the hindbrain). Segmentation of the latter leads to the formation of transient structures called rhombomeres. The division of the hindbrain into rhombomeres underlies a functional subdivision necessary for neuronal populations differentiation and axons projection as well as for neural crest determination and migration (for review, see Guthrie, 2007). Hindbrain segmentation occurs independently from the PSM segmentation. Segmentation of the mesodermal derivative, the PSM, is called somitogenesis. In chordates, the PSM lies on each side of the midline, thus bilaterally to the notochord, axial organs and neural tube.

Somitogenesis is the process by which vertebrate embryos acquire a segmented structure (reviewed in Saga and Takada, 2001). In the following, I will only focus on data concerning trunk segmentation excluding the hindbrain.

Trunk segmentation: Somitogenesis.

The pattern of the segmentation of the PSM is established during embryogenesis through the production of initially functionally equivalent units, the somites. Somites are transient embryonic structures that will give rise to the vertebrae, skeletal muscles and skin derivatives and provide a scaffold for assembly of the peripheral nervous system (Buckingham, 2006; Holloway and Curie, 2005). The somitogenesis process begins during gastrulation, and continues during subsequent axis elongation. The first pair of somites forms immediately caudal to the otic vesicle. In contrast to the fly embryo in which segments arise simultaneously, vertebrate segmentation is a sequential process that proceeds synchronously while the embryo extends posteriorly The total number of somites produced is tightly set within a given species but can vary dramatically between species (Gomez *et al*., 2008; Woltering *et al.*, submitted). The pace of somite formation is also species-specific: formation of one pair of somites takes 90min in chick, 120min in mouse, 30 min in zebrafish, and about 45min in *Xenopus*.

In *Xenopus laevis*, morphological somite formation begins at late neurula stage (stage 17) and continues until swimming tadpole stage (stage 40) when about 45 pairs of somites have formed (Nieuwkoop and Faber, 1956). Moreover, in *Xenopus* somites are laid down asynchronously on each side of the midline: the somite on the right side forms slightly earlier than the one on the left, reminiscent of the somitogenesis in Amphioxus. But the overall somites are symmetrically placed along the A-P axis. Furthermore, somitogenesis in *Xenopus* is different from its vertebrate counterparts in the mode of somite formation. In chicken and mouse somites are being added sequentially and synchronously on both sides of the neural tube, by budding off of epithelial spheres of cells (reviewed in Kulesa *et al*., 2007; Pourquié, 2003) while in *Xenopus*, a group of cells of the anterior PSM separate from the unsegmented tissue by the means of a fissure; then these cells (initially mediolaterally positioned) undergo a 90° rotation (ending anterior-posteriorly oriented) to achieve their final position (Fig. 4; Afonin *et al*., 2006; Kulesa *et al.*, 2007).

The symmetry of segmentation of the PSM seems to be controlled by RA signaling events as it has been recently pinpointed in chick, zebrafish and mouse (Fig. 4; for review see Duester, 2007; Sirbu and Duester, 2006; Kawakami *et al.*, 2005; Vermot *et al*., 2005; Vermot and Pourquié, 2005).

Somites are embryonic structures that will give rise to several “adult tissues”. Thus, a somite will ultimately give rise to the myotome (muscle precursor), sclerotome (bones and derivatives presursor) and dermatome (skin and derivatives). *In Xenopus*, the

ultimate structures generated by the myotome are mainly muscles (Fig. 4; Brand-Saberi and Christ, 2000; Jen *et al.*, 1997).

Classical embryology experiments revealed that periodicity and directionality of somite formation is an intrinsic feature of the cells within the PSM. Indeed, a rostro-caudal inversion of the PSM does not affect the formation of the somites in the inverted region, which maintain their original direction (Christ *et al.*, 1974). Moreover, isolation of the PSM from the surrounding tissue does not affect the segmentation process (Palmeirim *et al.*, 1998). This led to the idea of a "somitogenesis clock" working autonomously in the PSM cells (Stern and Vasiliauskas, 2000; reviewed in Dale and Pourquié, 2000). Cooke and Zeeman proposed the first model of segmentation known as "Clock and wavefront" model (Cooke and Zeeman, 1976). This model postulated the existence of a positional information gradient along the A-P axis of vertebrate embryos (the wavefront), which interacts with a cellular oscillator (the clock) to set the time and space at which a somite is formed (reviewed in Kulesa *et al.*, 2007). Remarkably, a couple of decades after this model has been proposed, the functional importance of the clock and the gradient have been confirmed experimentally

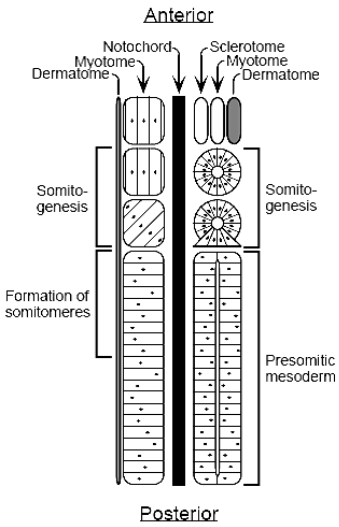

Figure 4: **Modes of somites formation and their derivatives.** In mouse and chick, somites form by budding off from the PSM and making epithelial spheres (right on the picture). Epithelial somites will differentiate into skin derivatives (dermatome), muscle derivatives (myotome) and bones derivatives (sclerotome). *Xenopus* shows a peculiar mode of segmentation. Anterior PSM cells detach from the posterior PSM by fissures. In addition, they undergo a 90° rotation to end up in a anterior-posterior position, parallel to the neural tube and notochord. Skin precursor, the dermatome, forms a sheet along the somites and PSM, whilst somites will differentiate mainly in muscle derivative (myotome). Scheme from Jen *et al.*, 1997

The clock.

A somitogenesis clock has been identified in chick, mouse, zebrafish and frog, and consists of oscillatory genes expression within the PSM (Forsberg *et al.*, 1998; McGrew *et*

al., 1998; Holley *et al*., 2000; Jiang *et al*., 2000; Jouve *et al.*, 2000; Sawada *et al*., 2000; Bessho *et al*., 2001; Li *et al.*, 2003). This indicates that the segmentation clock is conserved among vertebrates. Although the PSM does not exhibit any morphological segmental pattern, it is endowed with periodic information as it shows rhythmic expression of particular genes, known as the cycling genes (Pourquié, 2003, 2001). Most of these genes are involved in Notch signaling suggesting that Notch activation plays a critical role in the oscillations; thus, *Notch1* knockout exhibit segmentation phenotype placing Notch signaling at the core of the PSM oscillations (clock) (Hayward *et al.*, 2008; Rida *et al.*, 2004).

The Notch pathway has been proposed to generate oscillations by forming negative feedback loop with its modulator Lunatic fringe (Lfng) where the protein down regulates its mRNA expression (reviewed in Cinquin, 2007; Bessho and Kageyama, 2003). However, somites still form when Notch signaling is impaired or abolished, suggesting that additional factors must be involved. For instance, in mouse lacking RBPjk (Notch effector), somites do form in an irregular manner and with some delay (Oka *et al.*, 1995). Thus, either compensatory mechanisms exist or Notch is not the clock trigger element of the segmentation.

Another signaling pathway has been shown to exhibit oscillating behavior within the PSM: Wnt/β-catenin is cyclic in mouse PSM and induces cyclic transcription of Axin2, a Wnt signaling inhibitor (Aulehla *et al.*, 2003). However, Axin2 mutants do not show somitogenesis defects (Yu *et al.*, 2005). Moreover, there is persistence of Axin2 oscillations in the Notch pathway *Dll1* mutant (Aulehla *et al*., 2003) indicating independent expression of Axin2 from the Notch pathway.

The existence of two signaling pathways, each producing oscillations of their targets gene activity as a result of negative feedback mechanisms, and each linked to segmentation clock, opens the question of whether they exist in parallel or interact. Cross talks between the pathways involved in the somitogenesis clock has been extensively investigated and nicely reviewed by Cinquin (Cinquin, 2007; Dev Dyn special issue, 2007).

The wave.

Early segmentation models suggested the existence of a morphogen gradient peaking at the caudal end of the embryo, the “wavefront”, a maturation wave involved in positioning somites along the A-P axis of the embryo (Cooke and Zeeman, 1976; Meinhardt, 1986). Three major pathways RA, FGF and Wnt are known to influence the position of the somite boundaries in the PSM (Fig. 5; Cinquin, 2007).

Pourquié's lab has shown a graded distribution of FGF8 mRNA and protein along the PSM with the peak in the tailbud (Dubrulle and Pourquié, 2004). Local increase of FGF8 protein in the PSM triggers the formation of smaller somites anterior to the FGF bead, and bigger somites posterior to it. Blocking FGF signaling gave the opposite phenotype, and triggers the formation of larger somites. This strongly supports the importance of FGF8 in determining the position at which the segment boundary will form. A similar role of FGF in positioning somites boundaries has also been reported in the case of zebrafish, where a FGF gradient appears (Sawada *et al*., 2001).

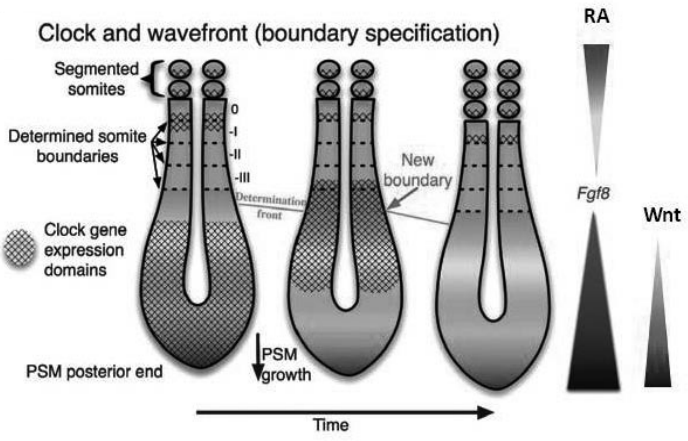

Figure 5: **Somitogenesis in vertebrate involves a clock and a wavefront.** Clock genes oscillate through the PSM from posterior to anterior during body elongation (grid pattern). This cyclic pattern
marks within the anterior PSM the site where a somite will form. FGF, Wnt and RA gradients are believed to shape the maturation wavefront (adapted from Cinquin, 2007).

RA synthesized in already formed somites diffuses towards the posterior of the embryo, and counteracts FGF signaling, thus impacting the position of the somite boundary (Moreno and Kintner, 2004; Diez del Coral *et al.*, 2003). Treatment of *Xenopus* embryos with RA triggers the formation of larger somites (Moreno and Kintner, 2004) while quail embryos deficient in vitamin A (and thus RA deficient), exhibit small somites (Maden *et al.*, 2000).Furthermore, mice embryos depleted of RA also show smaller tightly packed somites, underlying the importance of a proper balance of FGF and RA signals (Shiotsugu *et al.*, 2004; Niederreither *et al*., 1999).

The existence of a Wnt gradient has been suggested by the work of Aulehla and co-workers. The inhibitor of the Wnt pathway, Axin2 shows a graded mRNA expression

along the PSM with the peak situated posteriorly (Aulehla *et al.*, 2003). Axin2 is also a canonical Wnt target and its graded expression suggests the existence of a Wnt gradient in the mouse PSM. As in the case of FGF8, local increase of Wnt3a leads to the formation of small somites. Interestingly, the expression of FGF8 RNA is strongly down-regulated in the tail bud of the Wnt3a mutants, suggesting that FGF8 signaling is dependent on Wnt activity (reviewed in Aulehla and Hermann, 2007; Aulehla *et al.*, 2003).

A special issue of Developmental Dynamics (June, 2007) is dedicated to summarize data known to date about segmentation.

Integration of segmentation and body elongation.

The process of segmentation is intimately linked to the formation of the anterior-posterior axis, and the pace of the somite formation is tightly correlated to the axis elongation (Fig. 5). Despite their common origin, somites at different positions along the anterior posterior axis will give rise to differently patterned structures, as is easily illustrated by the different shapes of vertebrae in the axial skeleton. *Hox* genes are key players in determining these differences in morphological structures along the A-P axis (Deschamps *et al*., 1999).

Hox genes themselves appear to oscillate in the mouse PSM along with the segmentation genes. Interestingly, Zákány and co-workers have shown that *Hox* genes expression is dramatically down-regulated in the PSM and in the forming somites of the Notch pathway mutant, RBPkj, thus providing a link between Notch pathway and *Hox* genes (Zákány *et al.*, 2001). Furthermore, analysis of Notch pathway mutants revealed phenotypes resembling homeotic mutations (for example the Notch1 null mutant, Cordes *et al.*, 2000).

Recently, in *Xenopus*, the knockdown of *X-Delta-2* (a Notch ligand) triggers somitogenesis defects (Peres *et al*., 2006). The same report has shown that *X-Delta-2* and *Hoxd1* (the earliest *Hox* gene expressed in the frog) are expressed in overlapping domains during gastrulation. Moreover, knocking down *X-Delta-2* leads to downregulation of several *Hox* gene expression during gastrulation and neurulation (*Hoxb4*, *Hoxc6* and *Hoxb9*). Interestingly, knockdown of paralogous group 1 *Hox* genes leads to the downregulation of *X-Delta-2* and results in related segmentation defects. These data suggest the existence of a strong bidirectional link between Notch signaling (thus segmentation) and *Hox* genes (thus A-P patterning). In light of these data, we investigated the potential function of other *Hox* genes in the frog somitogenesis process.

Thus, in chapter V, we present data demonstrating the requirement for another *Hox* gene in *Xenopus* segmentation.

Aim of this thesis

The processes of gastrulation, neural induction and somitogenesis, which take place during early embryogenesis, are crucial for the development of a complete and functional organism. This thesis aims to elucidate the roles of the family of *Hox* transcription factors during these processes and early development of *Xenopus laevis* in general. An extensive body of literature already exists with respect to the developmental roles of the *Hox* genes but, due to the complicated organization of this gene family and redundancy (there are 39 *Hox* genes in tetrapods) their functions are still surrounded by many uncertainties.

As described above, the *Hox* genes are expressed in a highly structured pattern along the anterior-posterior body axis. In Chapter II, we investigate processes that are necessary for the establishment of these spatially colinear *Hox* expression zones along the A-P axis during gastrulation. Previously, it was shown that in absence of the Spemann organizer, embryos develop without a clear anterior-posterior pattern of *Hox* expression (Wacker *et al.* 2004a). Which of the different functions of the Spemann organizer is involved in the establishment of this pattern has not been known. In chapter II, the different functions of the Spemann's organizer were experimentally dissected by ablation of total organizer function and subsequent restoration of individual functions. Thereby, it was shown that neural induction is the function required for the induction of the *Hox* AP pattern.

The functional role of specific *Hox* genes during the patterning of the neural plate was investigated in chapter III and chapter IV by morpholino knockdown. In chapter III, the *Hoxc6* gene was knocked down and a requirement for proper induction of motor neuron pools is shown. In absence of Hoxc6 a significant lower number of primary motor neurons develops in the neural tube. In Chapter IV, the function of the Hox-1 paralogous group genes in patterning the hindbrain is investigated. There are three paralogous 1 members, namely *Hoxa1*, *Hoxb1* and *Hoxd1*. In the mouse, loss of function experiments by knockout of *Hoxa1* and *Hoxb1* have already demonstrated a function in patterning individual hindbrain rhombomeres. Due to the high redundancy in patterning functions for paralogous groups, we were interested to investigate an experimental triple loss of function. Double knockdowns reveal that the functions of *Hoxa1* and *Hoxb1* are conserved between mouse and *Xenopus*. The triple *Hox1* knockdown (including *Hoxd1*) reveals an extremely highly redundant patterning function for this group of genes, as only the knockdown of all three

genes results in dramatic defects in hindbrain patterning. It turns out that in absence of Hox1 group, the hindbrain acquires a rhombomere 1 state and that *Hox1* group genes are thus involved in patterning of the hindbrain posterior to rhombomere 1. We further observed a defect of hindbrain derived neural crest migration, leading to severe craniofacial developmental defects.

Chapter V investigates the role of *Hoxc6* during segmentation of the Xenopus embryo. We show a functional role of *Hoxc6* genes in trunk segmentation. In the absence of *Hoxc6*, *Xenopus* embryos fail to undergo proper trunk segmentation although axis extension is not affected. These results closely connect to previous work showing that *Xenopus* embryos have defects in trunk segmentation in the *Hox1* knockdown. We speculate that the previously observed *Hox1* phenotype occurs through regulation of *Hoxc6* and that *Hoxc6* acts through directly regulating *X-Delta2*.

The first site of *Hox* gene expression in the *Xenopus* embryo is the ventro-lateral mesoderm, during gastrulation. Slightly later, expression of the genes appears in the neurectoderm, where they are ultimately expressed in a spatial colinear sequence (Wacker *et al*., 2004a). The onset of *Hox* expression in the mesoderm and in the neurectoderm appears in a highly synchronized fashion which could be indicative of a causal relationship. In Chapter VI the phenomenon of *Hox* gene transfer is investigated and a possible mechanism relaying positional information from the ventro-lateral mesoderm to the overlying neurectoderm is proposed. In experimental explants settings, using the wrap assay (Jansen *et al.,* 2007), we show a requirement for *Hox* gene expression in the mesoderm for the expression of the same gene in the physically connected neurectoderm. The expression of a *Hox* gene in non-*Hox* expressing mesoderm is sufficient to induce expression of the same gene in overlying neurectoderm.

In summary, this thesis describes the functions of *Hox* genes during early development of *Xenopus laevis*. It reports on the investigation of the mechanisms responsible for establishing an anterior-posterior pattern of *Hox* gene expression (chapter II and VI) and the function of *Hoxa1*, *Hoxb1*, *Hoxd1* (chapter IV) and *Hoxc6* (chapter III and V) within these expression domains during neurogenesis and somitogenesis.

Chapter 2

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

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Abstract

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

Keywords: Xenopus; Anterior–posterior patterning; Organizer; Neurectoderm; Activation; Transformation; Hox

1. Introduction

In all vertebrates, the formation of the main body axis begins with the generation of an organizing centre. Interactions between this organizing centre and surrounding tissues during gastrulation generate a basic body plan. Since the initial discovery of this structure in the dorsal blastopore lip of amphibians (the Spemann organizer, hereafter called the organizer (Spemann and Mangold, 1924)), comparable organizing centers have been found in other vertebrate groups (i.e. the node in mouse, Hensen's node in chicken, embryonic shield in zebrafish (Joubin and Stern, 2001; Niehrs, 2004). Many of their functions have been identified and the molecular pathways involved have been characterized. In the amphibian *Xenopus laevis* the functions of the organizer have been divided into three categories (Harland and Gerhart, 1997). First, self-differentiation of the organizer generates a variety of mesodermal and endodermal tissues, including head mesoderm, notochord, and pharyngeal endoderm. Second, the organizer performs morphogenetic movements and in addition induces them in adjacent cells (e.g. convergence and extension in the presumptive notochord and in the somitic mesoderm). The timing of mesodermal and endodermal internalization also depends on signals from the organizer. Bottle cell formation, involution and vegetal rotation start up to 2 h earlier on the organizer side than on the opposite side (Shih and Keller, 1994; Ibrahim and Winklbauer, 2001; Winklbauer and Schürfeld, 1999). Third, the organizer secrets signals which affect all three germ layers of the developing embryo.

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Most of these signals have been found to antagonize ventralizing signals like BMPs, Wnts, and Nodals (for review Niehrs (1999); De Robertis and Kuroda (2004)).

It has been clear for a long time that the organizer is of crucial importance for the development of the anterior–posterior (AP) axis (Spemann and Mangold, 1924). In an early model, it was postulated that different portions of the organizer mediate different positional values along the AP axis (Mangold, 1933; Eyal-Giladi, 1954). Head, trunk, and tail organizing areas have been described in the Xenopus organizer (Zoltewicz and Gerhart, 1997; Lane and Keller, 1997) and other vertebrates (Agathon et al., 2003; Kaneda et al., 2002). Vertical signals from different portions of the internalized organizer mesoderm to the overlying prospective neurectoderm have been suggested for regulation of a few, very anterior patterning genes (Hemmati-Brivanlou et al., 1990; Blitz and Cho, 1995). In addition, gradients of secreted molecules (FGFs, Wnts, retinoic acid) have been postulated to act in a planar way along the AP axis to define more posterior values (Cox and Hemmati-Brivanlou, 1995; Lamb and Harland, 1995; Kiecker and Niehrs, 2001; Durston et al., 1989).

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

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

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

To get a better understanding of the role of the organizer in AP patterning we dissected the organizer functions and manipulated them separately. Since several molecular pathways involved in different functions of the organizer are well characterized, single organizer functions can be knocked down in certain tissues or whole embryos. Alternatively, the formation of an organizer can be efficiently prevented by UV irradiation (Scharf and Gerhart, 1983). A single organizer function can then be restored without reestablishment of others.

In this paper, we demonstrate that neural activation is the function of the organizer, which is crucial for the establishment of a detailed AP pattern. Prevention of neural activation by localized application of the constitutively active BMP receptor *hAlk-6* disables the expression of AP patterning genes. Restoration of neural activation by localized application of the dominant inhibitory BMP receptor tBr and *FGF-4* under conditions, where all other organizer functions are absent, i.e. ventralized embryos or on the ventral side of normal embryos, enabled the expression of AP marker genes. We conclude that neural activation by the organizer makes ectodermal cells competent to respond to the transforming AP patterning signals originating from the non-organizer mesoderm, thereby enabling the stable formation of posterior AP positional values, which are characteristic for the trunk.

2. Results

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

We have recently published the observation that organizer mesoderm alone is not sufficient to induce the expression of posterior positional markers in the neuroectoderm, indicating that neural transformation is disturbed or even absent (Wacker et al., 2004a). Here we use the so-called wrap assay to analyze in detail the effects of the organizer on AP patterning of the ectoderm. For this wrap assay,

pieces of mesoderm are recombined with ectoderm (Fig. 1A). After several hours these wraps are fixed, used for in situ hybridization and then bisected across the mesodermal implants (Fig. 1B, C). Due to tissue separation (Wacker et al., 2000), mesodermal and ectodermal cells do not intermingle during the experiment as is shown here with fluorescence labeling (different mesodermal tissues were labeled in red and green using converted and not converted EosFP (Wiedenmann et al., 2004; Wacker et al., 2007) and then implanted into unlabeled ectoderm, Fig. 1C).

For our purpose, pieces of organizer mesoderm were wrapped in ectodermal animal caps. This exclusive recombination of the donor and the recipient of neural activation signals reduces interfering effects from other sources. Wraps were analyzed by in situ hybridization for pan-neu-

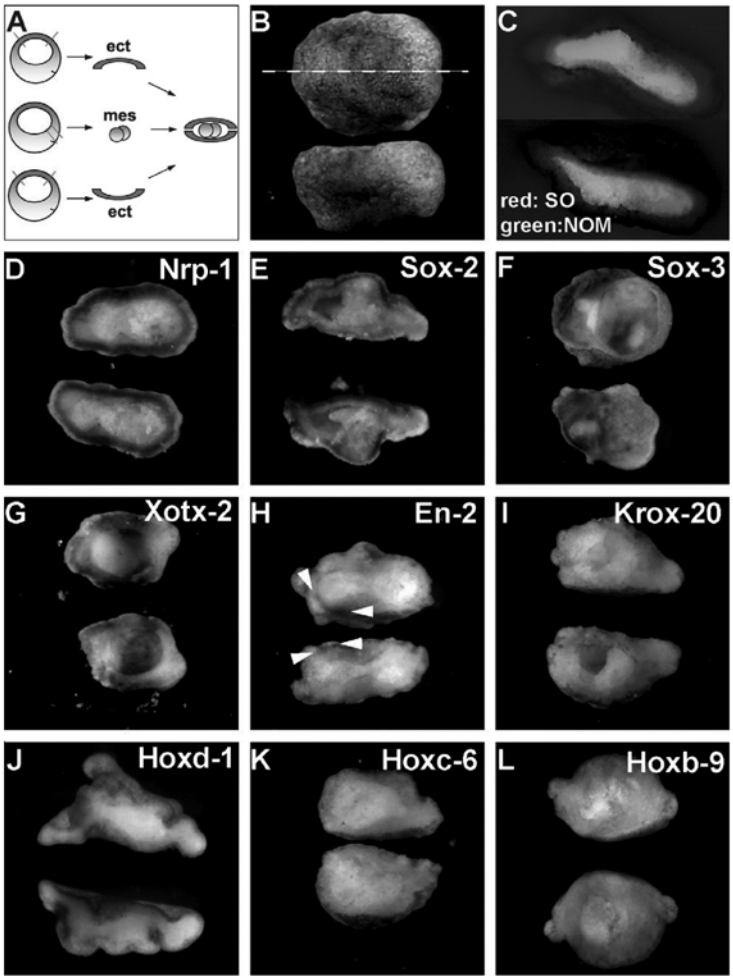

Fig. 1. The Spemann organizer induces a rudimentary AP pattern in wrap assays. (A) In the wrap assay small mesodermal explants (mes, e.g. organizer or non-organizer mesoderm) are wrapped into ectodermal animal caps (ect) to analyze inductive events. (B) A wrap in topview and lateral view. For the analysis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

ral markers and AP patterning genes, at a time when sibling embryos were at neurula stages. A set of pan-neural markers was expressed in the ectoderm of the wraps indicating that neural tissue was induced (*Nrp-1*, *Sox-2*, *Sox-3*, Fig. 1D–F). In addition, the anterior gene *Xotx-2* is expressed (Fig. 1G). *En-2*, which is normally detected in the midbrain–hindbrain border, is expressed rudimentarily (Fig. 1H). More posterior genes that are normally expressed in the hindbrain or in the spinal cord were not found. This was true for the hindbrain marker *Krox-20* (Fig. 1I), and a set of Hox genes, representing different AP positions (*Hoxd-1*, *Hoxc-6*, *Hoxb-9*, Fig. 1J–L).

We conclude that in wraps, which contain exclusively organizer mesodermal implants, neural tissue of anterior quality is induced, whereas positions behind the midbrain are absent, indicating a failure of neural transformation. This is in correspondence with the observation that transforming non-organizer signals are necessary for AP patterning.

2.2. An efficient knock down of the organizer by UV irradiation

UV irradiation has been found to be an efficient way of ventralizing Xenopus embryos. Prevention of cortical rotation results in a block of the dorsal Wnt pathway and of subsequent organizer formation (Larabell et al., 1997; Scharf and Gerhart, 1983; Vincent and Gerhart, 1987). UV irradiation may therefore be used to obtain embryos lacking an organizer. We characterized these ventralized embryos for aspects of gene expression and aspects of tissue movements to check the absence of the organizer and of its functions. Changes in morphogenesis have been reported in UV-ventralized embryos (Mise and Wakahara, 1994). Using lineage labeled embryos, we analyzed, if, except for the organizer specific defects, gastrulation movements still take place. Especially involution, which places mesoderm underneath the ectoderm, might be important for AP patterning. In embryos expressing the convertible fluorescent protein EosFP, a portion of the mesodermal marginal zone was converted from green to red fluorescence at the beginning of gastrulation, as has been described previously (Wacker et al., 2007). These embryos are shown at an early gastrula stage (Fig. 2A, B) and at a late gastrula stage (Fig. 2C, D). The involution of mesoderm takes place in both, in non-treated and UV-treated embryos. Cross sections demonstrate that the labeled mesoderm is internalized in both during gastrulation (Fig. 2G, H). Differences were found for the organizer related convergence and extension. Whereas in non-treated embryos the labeled spot elongates extensively (corresponding to convergence and extension of the notochordal precursors, Fig. 2C, G), this was dramatically reduced in UV-treated embryos (Fig. 2D, H). The effect on blastopore formation and closure was also organizer related. In non-treated embryos the blastopore first forms on the dorsal side and then consecutively in lateral and ventral regions. In UV-treated embryos the blastopore appeared as a complete ring at the time, when it formed on the ventral side in non-treated embryos. The blastopore then closed at about the same time in both, non-treated and UV-treated embryos (not shown). We conclude that, except for the organizer specific morphogenetic movements, gastrulation movements in UV-treated embryos are normal.

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

Overall we find that organizer independent prerequisites for AP patterning (i.e. non-organizer mesoderm formation and appropriate morphogenetic movements, see also Wacker et al. (2004a)) were not affected by the UV treatment, whereas organizer specific genes and functions were absent. This delivers the optimal experimental preconditions for our following experiments.

2.3. Neural activation enables AP patterning by transformation without an organizer

One of the organizer functions is neural activation. To study the importance of this function for AP pattern formation, we prevented formation of the organizer and thereby its functions by UV irradiation of early embryos. We then neurally activated their ectoderm by injection of a combination of mRNAs encoding for a dominant negative BMP receptor (tBr) and a very low dose of *FGF-4*. This treatment suppressed epidermal fate in the ectoderm and efficiently promoted a neural fate (Linker and Stern, 2004; Delaune et al., 2004). To confirm that the injected mRNAs exclusively led to neural activation, we injected the same amounts of either tBr or *FGF-4* mRNAs alone. This gave the same results as described in Delaune et al. (2004). Reduction of BMP signaling caused ectoderm to adopt a neural crest fate, very low doses of *FGF-4* mRNA

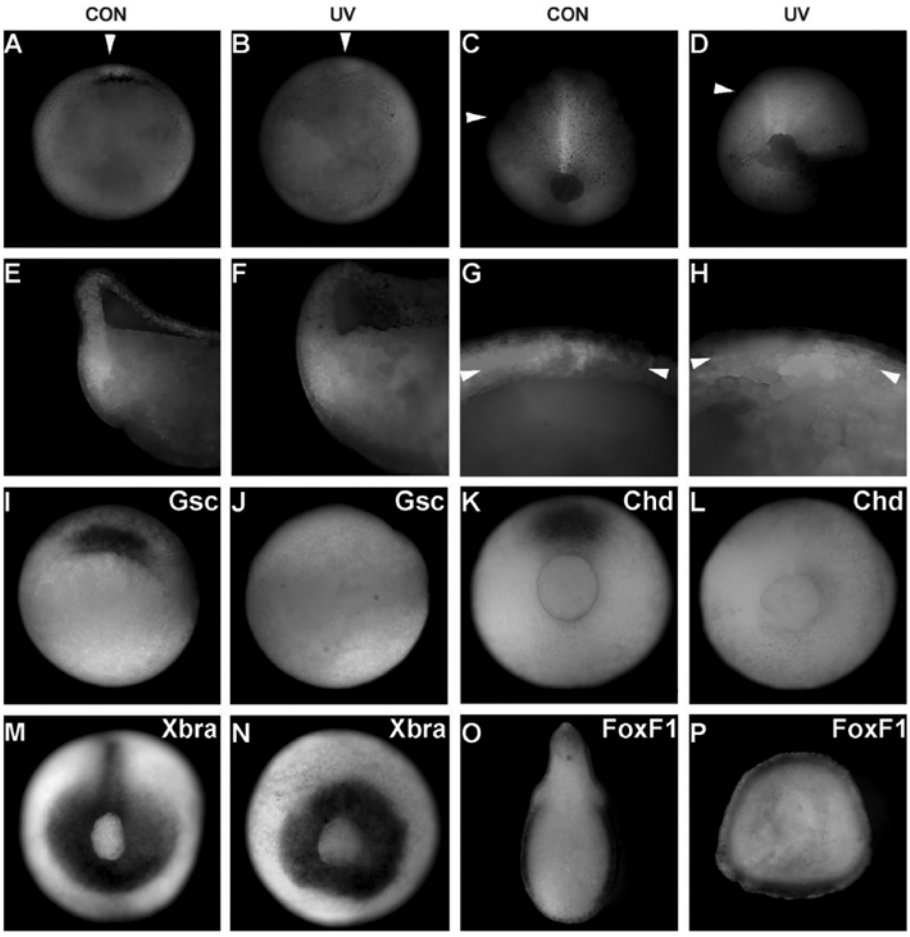

Fig. 2. UV treatment results in embryos without an organizer. Embryos were injected with EosFP. The EosFP in a small region of the marginal zone was converted at the beginning of gastrulation. (A) An untreated control embryo (CON) with a conversion in the organizer region. (B) An UV-treated embryo (UV)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

did not cause mesoderm induction or posteriorization (not shown).

Embryos treated in this way were analyzed for mesodermal and ectodermal marker gene expression as well as for markers that show AP patterning. Since completely UV-ventralized embryos do not have an organizer–non-organizer pattern, the resulting effects should be radially symmetric and can therefore be distinguished from the effects caused by partial ventralization, which should still show an organizer–non-organizer asymmetry. The absence of organizer both in ventralized embryos and in ventralized and neurally activated embryos, was confirmed by the reduction of staining of the organizer genes *Goosecoid* at early gastrulation (Fig. 3A–C) and of *Chordin* at later

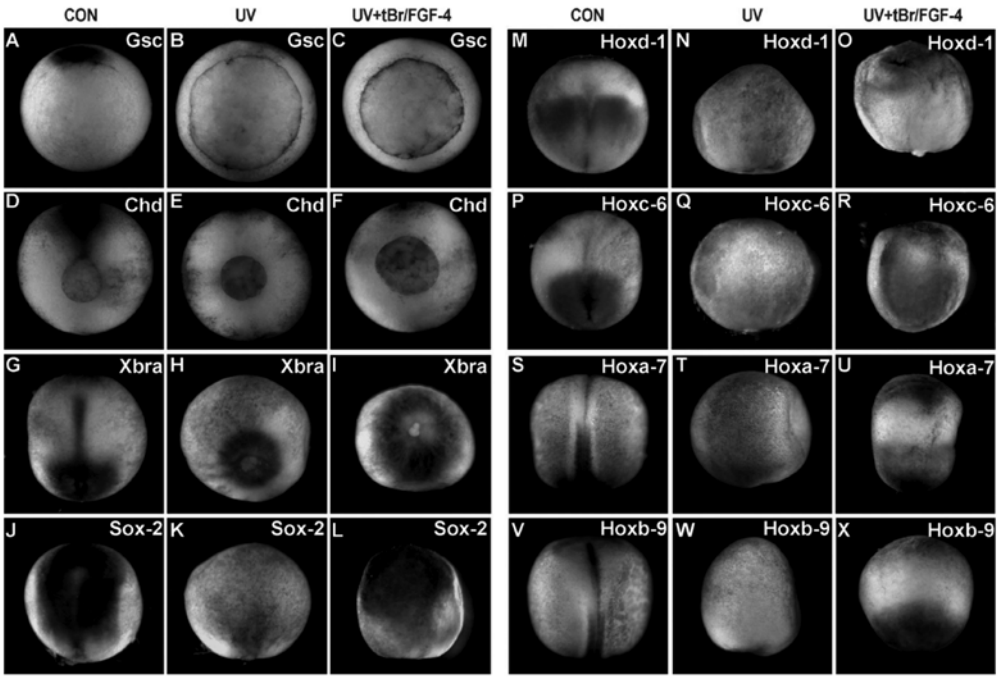

Fig. 3. Restoration of neural activation by injection of tBr and *FGF-4* in predominantly ectodermal precursors of UV-treated embryos results in AP patterning of embryos without Spemann organizer. The first column shows expression of different markers in untreated control embryos (CON). The second column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

gastrulation (Fig. 3D–F). The *Xbra* expression pattern supported this, since the presumptive notochordal *Xbra* expression is absent in both, UV-treated embryos and UV-treated embryos injected with tBr and *FGF-4* mRNAs (Fig. 3G–I). The non-organizer mesodermal expression domain of *Xbra* is still present and even expanded in ventralized and neurally activated embryos, demonstrating that there was no reduction of non-organizer mesoderm (Fig. 3G–I). The ectoderm was analyzed for the expression of the neural marker *Sox-2*. Neural fate, which is absent in UV-treated embryos, is brought back by the injection of tBr and *FGF-4* mRNAs (Fig. 3J–L). However, organizer–non-organizer asymmetry is not restored, since *Sox-2* has a radially symmetric expression. Concurrently the expanded epidermal fate (analyzed with XK81A1) in UV-treated embryos is radially suppressed by additional injection of the animal cap with the mRNAs for tBr and *FGF-4* (not shown).

AP patterning, which is absent in UV-treated embryos, is restored in UV-treated embryos after injection of tBr and *FGF-4* as the expression of Hox genes shows. The expression patterns of four different Hox genes (*Hoxd-1*, *Hoxc-6*, *Hoxa-7*, and *Hoxb-9*) were compared in non-treated embryos, in UV-treated embryos, and in UV-treated and tBr- and FGF-4-injected embryos. The characteristic patterns of these Hox genes along the AP axis are shown in non-treated embryos at early neurula stages (Fig. 3M,

P) and late neurula stages (Fig. 3S, V). After UV irradiation, Hox gene expression is drastically reduced or absent (Fig. 3N, Q, T, W). The injection of mRNAs for tBr and *FGF-4* into ectodermal precursors enables UV-ventralized embryos to express these Hox genes again in their normal AP order (Fig. 3O, R, U, X). In contrast to non-treated embryos, the expression patterns are not restricted to the dorsal side, but appear in a radially symmetric pattern at their correct positions along the AP axis.

These results are supported by experiments using wrap assays. A wrap containing both, organizer mesoderm and non-organizer mesoderm expresses Hox genes in the surrounding ectoderm (shown for *Hoxd-1*, *Hoxd-4*, and *Hoxb-9* in Fig. 4A–C). Absence of the organizer in such wraps disables the expression of these Hox genes (Fig. 4D–F). This can be rescued by the injection of tBr and *FGF-4* to get neural activation without an organizer (Fig. 4J–L), even though the injection of these neuralizing

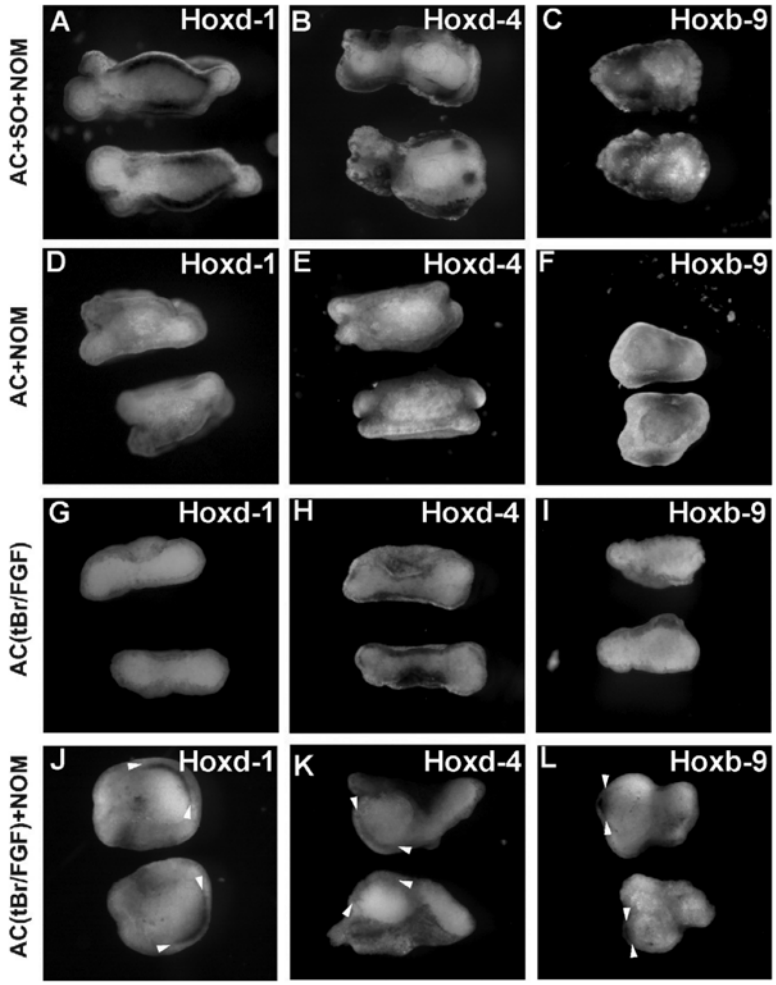

Fig. 4. The Spemann organizer function for AP patterning in wrap assays can be replaced by neural activation. (A–C) Wraps containing both, organizer and non-organizer mesoderm (AC + SO + NOM) express *Hoxd-1* (A), *Hoxd-4* (B) and *Hoxb-9* (C) in the ectoderm. (D–F) Wraps containing exclusively non-organizer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

factors does not result in Hox gene expression in the absence of mesoderm (Fig. 4G–I).

To further test the importance of neural activation to enable the expression of AP positional markers, we ectopically expressed tBr and *FGF-4* in embryos as far away from the organizer as possible. For this purpose, we injected these mRNAs at the 32-cell stage into the A4-blastomere (i.e. animal tier, opposite to the prospective organizer). Using GFP for lineage tracing, we selected embryos at late neurula stages, which showed the label exclusively outside of the central nervous system (Fig. 5A). Ectopic *Sox-2* expression indicates that ectopic neural activation has taken place (Fig. 5B). In such embryos domains of ectopic Hox gene expression were detected as it is shown for *Hoxd-1* (Fig. 5C), *Hoxd-4* (Fig. 5D), *Hoxc-6* (Fig. 5E), and *Hoxb-9* (Fig. 5F). In tadpoles selected for a ventral localization of the GFP label (Fig. 5G), the coinjection of tBr and *FGF-4* resulted in

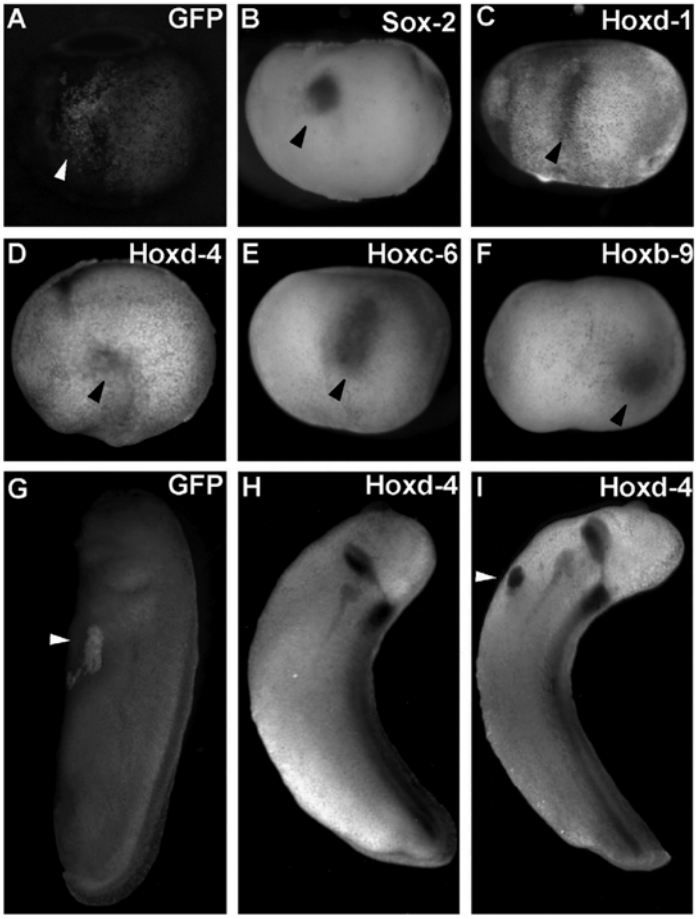

Fig. 5. Ectopic areas of neural activation after injection of tBr and *FGF-4* in a ventral animal blastomere at 32-cell stage show AP patterning gene expression independent of the organizer. (A, G) Lineage tracing with GFP (arrowheads) after coinjection of the mRNAs of tBr, *FGF-4*, and *EGFP*. (A) Ventrolateral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

small ectopic expression domains (shown for *Hoxd-4*, Fig. 5I) compared to embryos GFP-injected without these mRNAs (Fig. 5H).

Overall, we find that neural activation independent of an organizer in UV-treated embryos, in wraps, and on the ventral side of normal embryos caused expression of AP positional markers. This demonstrates that AP patterning occurs independently of other organizer functions, when neural activation takes place.

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

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

As an alternative experimental approach we used the wrap assay to show that blocking neural activation in the ectoderm blocked expression of AP patterning genes, even though both organizer and non-organizer mesoderm are present. This approach also completely restricts the effect of *Alk6* injection to the ectoderm and excludes a direct effect of the injection on the mesoderm. We injected *hAlk-6* mRNA into whole embryos to disable neural activation. Ectodermal animal caps from these embryos were used to make wraps containing both organizer and non-organizer mesoderm from non-injected embryos. Wrap assays containing both types of mesoderm normally express different Hox genes in the ectoderm (Fig. 7A, C, E). When *hAlk-6* expressing ectoderm was used to wrap the organizer and non-organizer mesoderm, the expression of Hox genes was completely disabled (Fig. 7B, D, F).

We conclude that prevention of neural activation in embryos and in wraps by expression of *hAlk-6* in the ectoderm disables AP patterning, even though the other organizer functions are still present.

3. Discussion

3.1. The organizer induces anterior parts of the AP pattern

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

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

Even though the organizer does not directly produce the signals responsible for neural transformation, our experiments demonstrate that at least one of its functions is necessary to enable the ectodermal expression of posterior positional markers (see above and Wacker et al. (2004a)). The absence of the organizer and its functions disables transformation in whole embryos (e.g. UV-ventralized embryos) and in wrap assays. To analyze which of the

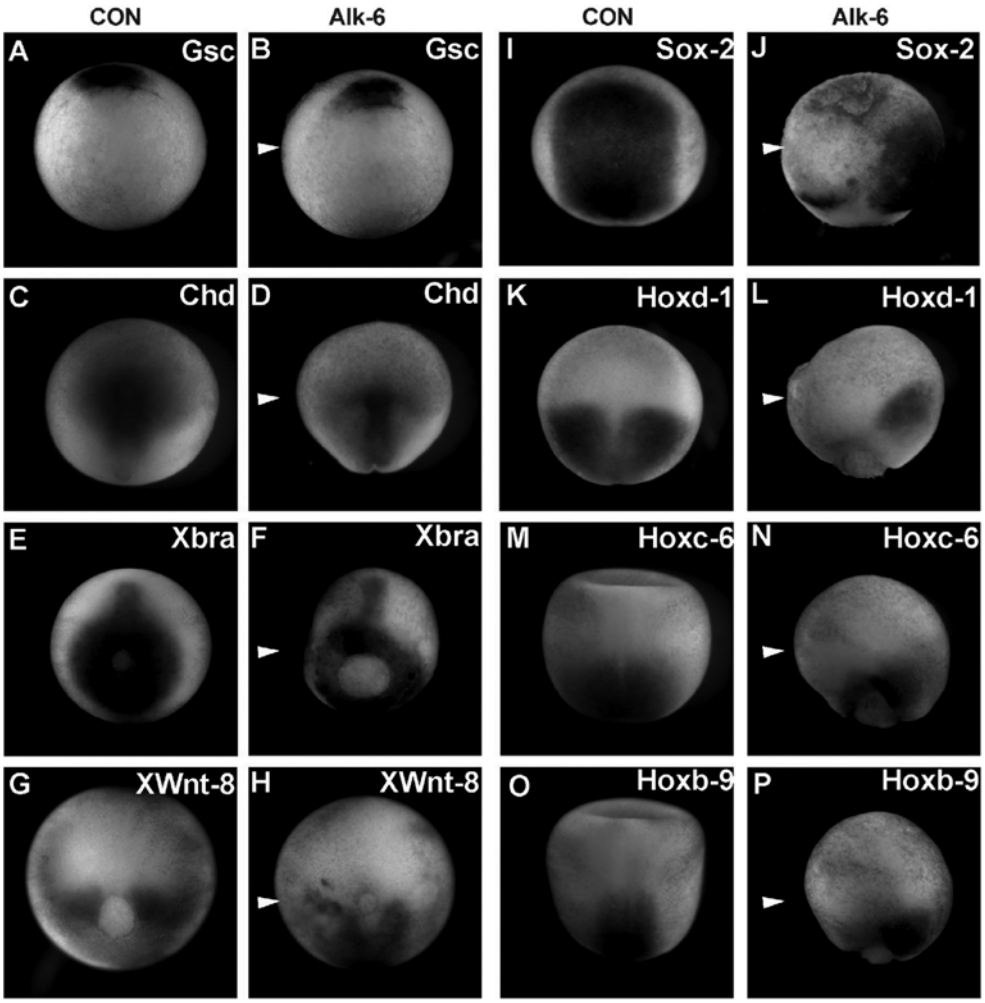

Fig. 6. Inhibition of neural activation in presence of an organizer strongly affects AP pattern formation. The mRNA of the constitutively active BMP receptor *hAlk-6* was injected into predominantly ectodermal precursors of the left side (arrowheads) to prevent neural activation on one side of the embryos. (A, B) Presence of the early *Gsc* expression in a non-injected control embryo (CON) and in a *hAlk-6* injected embryo (Alk-6) demonstrates that the formation of an organizer is not disturbed by the injection of *hAlk-6*. (C, D) Presence of *Chd* expression in a non-injected control embryo and in a *hAlk-6* injected embryo at the end of gastrulation demonstrates the remaining presence of the organizer during gastrulation. (E, F) Expression of the mesodermal marker *Xbra* in the organizer tissue of the presumptive notochord again shows the presence of the organizer both, in a non-injected control and in a *hAlk-6* injected embryo. (G, H) The posterior marker *Xwnt-8* remains in its domain on the injected side, indicating that *hAlk-6* expression does not prevent formation of posterior tissues. This is in accordance with the minorly affected posterior *Xbra* expression around the blastopore (E, F). (I, J) The neural marker *Sox-2* is expressed in the whole neural plate of control embryos, but it is drastically reduced after *hAlk-6* injection, indicating the inhibition of neural activation on the injected side. (G, H) Expression of *Hoxd-1*. (I, J) Expression of *Hoxc-6*. (K, L) Expression of *Hoxb-9*. The expression of all three Hox genes is reduced on the left side, where neural activation is inhibited by *hAlk-6* injection.

organizer functions is responsible for this, we manipulated the function of neural activation (also called neural induction or neuralization (Vonica and Brivanlou, 2006)) without affecting the other functions of the organizer. Neural activation was achieved by manipulating the BMP and FGF signal transduction pathways mainly in the ectoderm as has been described in the recent literature (Linker and Stern, 2004; Delaune et al., 2004). Enabling neural activation in the absence of the organizer leads to the formation of stable neural tissue, which can be transformed by signals

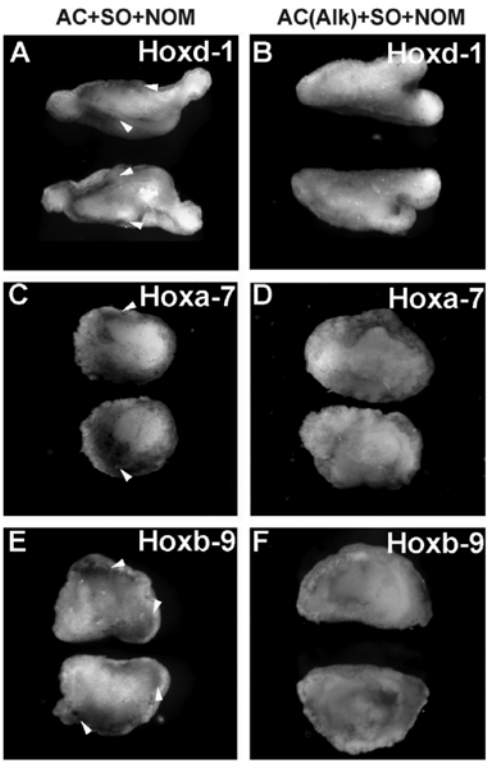

Fig. 7. Inhibition of neural activation by ectodermal expression of the constitutive active BMP receptor *hAlk-6* in wrap assays affects the ectodermal AP patterning. (A) Expression of *Hoxd-1* (arrowheads) in wraps containing both, organizer and non-organizer mesoderm surrounded by non-injected ectoderm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

not originating from the organizer. We have shown that disabling neural activation without inhibition of other organizer functions is sufficient to prevent the expression of posterior marker genes. Gain of function and loss of function experiments demonstrate that neural activation and stabilization by the organizer are necessary and sufficient to enable AP patterning of the trunk by organizer independent signals. The observation that under certain conditions AP patterning can occur in the absence of an organizer has been made in the mouse and zebrafish as well, although it was not clear, if the organizer was completely absent in these embryos (Ang and Rossant, 1994; Ober and Schulte-Merker, 1999).

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

Two sources of AP patterning signals resulting in expression of AP positional markers can be distinguished. First, the head portion of the organizer induces markers that are relevant for forebrain and midbrain, e.g. *Xotx-2* or *En-2* (above and Blitz and Cho (1995); Hemmati-Brivanlou et al. (1990)). Second, the non-organizer mesoderm has been described in different vertebrates as a signal source, establishing more posterior positional values of the trunk (i.e. hindbrain and spinal cord, for references see Section 1). For this second portion of the AP pattern, neural activation is an indispensable element. Ectopic neural activation independent of an organizer allows ectopic expression of AP patterning genes, if non-organizer mesoderm is present. The loss of neural activation prevents expression of AP patterning genes even in presence of both, the organizer and the non-organizer mesoderm.

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

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

3.4. Concluding remarks

The central question in this paper is the role of the Spemann organizer in AP patterning of the trunk. The organizer itself does not establish a complete AP pattern, but only a limited stretch from anterior to the midbrain–hindbrain boundary. Our experiments have shown that one essential function of the organizer for AP patterning of the trunk is to make the ectoderm competent to respond

to AP patterning signals from other sources. Our approach of bringing back single organizer functions into embryos without an organizer demonstrated that neural activation of the ectoderm is necessary and sufficient to provide this competence of the ectoderm to receive the signals that lead to transformation and formation of a complete and stable AP pattern in the trunk region of the body axis.

4. Experimental procedures

4.1. Handling and treating embryos

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

For lineage labeling the light convertible fluorescent protein EosFP was injected into whole embryos. Procedures for injection, conversion and analysis of this protein have been recently described (Wacker et al., 2007). At very early gastrulation the organizer or a corresponding area of the mesodermal marginal zone were converted from green to red. At the end of gastrulation whole embryos or bisected embryos were analyzed.

To prevent neural activation in ectodermal cells, 10 nl of *hAlk-6* mRNA (25 pg/nl) was injected in the two animal blastomeres on the left side of stage 4 embryos. To achieve neural activation, 10 nl of a mix of tBr (25 pg/nl) and *FGF-4* (0.004 pg/nl) was injected into the 4 animal blastomeres of UV-treated stage 4 embryos. Alternatively, 2 nl of this tBr/*FGF-4* mRNA mix was injected into the A4 blastomere (Dale and Slack, 1987) of a 32 cell normal embryo. To trace injections, GFP mRNA was coinjected. The mRNAs for injection were generated from plasmids: tBr64T, (dominant negative BMP receptor) (Graff et al., 1994); pCS2FGF-4 (made as described in Delaune et al. (2004)); pCS2ALK6HA (constitutively active *hALK6*) (kind gift from Peter ten Dijke, described in Wacker et al. (2004b)); pCS2EGFP; and pCS2+MT-d2EosFP (Wacker et al., 2007).

4.2. Wrap assays

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

For fluorescent labeled wraps embryos were injected with EosFP as described before (see above and Wacker et al. (2007)). At the beginning of gastrulation the EosFP in the organizer domain was converted to red. A piece of converted organizer mesoderm and a piece of labeled, but not converted, non-organizer mesoderm (green) were implanted in unlabeled ectoderm at early gastrulation and analyzed at early neurulation.

4.3. Detection of gene expression

Whole mount in situ hybridization (WISH) was performed as previously described (Wacker et al., 2004a). For wrap assays, embryos were cut with a razorblade after WISH. Antisense, DIG-labeled transcripts were prepared from the following plasmids: a 1109 bp *Sox-2* fragment in pBluescript SK(+); cDNA clone AGENCOURT_10482135 (*Sox-3*); pNPG152 (*nrp-1*) (Richter et al., 1990); pBS"HD-anti" (*Xotx-2*) (Blitz and Cho, 1995); a 1500 bp Engrailed-2 cDNA (*En-2*) (Hemmati-Brivanlou et al., 1991); a 1400 bp *Krox-20* fragment (Bradley et al., 1993); xHoxlab1 (*Hoxd-1*) (Sive and Cheng, 1991); cDNA clone XL094l20 (NIBB) (*Hoxd-4*);a 998 bp *Hoxc-6* fragment in pGEM1; Xhox-36.1 (*Hoxa-7*) (Condie and Harland, 1987); a 470-bp *Hoxb-9* fragment in pGEM3; pSP73Xbra (Smith et al., 1991); pCS2Chd (Sasai et al., 1994); pCS2Gsc (*Gsc*); pCSWnt8b (*Wnt8*) (Cui et al., 1995); pCS2FoxF1 (Köster et al., 1999); Brevican (*Xbcan*) (Sander et al., 2001); MyoD (Hopwood et al., 1989); a 807 bp fragment containing the ORF of *Xenopus Snail* cloned in pGEMT-easy; a 536 bp fragment from *XK81A1* in pGEM3 (Epidermal Keratin) (Jonas et al., 1989).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mod.2007.07.004.

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Chapter 3

Two *Hoxc6* transcripts are differentially expressed and regulate primary neurogenesis in *Xenopus laevis*

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Abstract

Hox genes are key players in defining positional information along the main body axis of vertebrate embryos. In *Xenopus laevis*, *Hoxc6* was the first homeobox gene isolated. It encodes two isoforms. We analyzed in detail their spatial and temporal expression pattern during early development. One major expression domain of both isoforms is the spinal cord portion of the neural tube. Within the spinal cord and its populations of primary neurons *Hox* genes have been found to play a crucial role for defining positional information. Here we report that a loss-of-function of either one of the *Hoxc6* products does not affect neural induction, the expression of general neural markers is not modified. However, Hoxc6 does widely affect the formation of primary neurons within the developing neural tissue. Manipulations of *Hoxc6* expression severely change the expression of the neuronal markers *N-tubulin* and *Islet-1*. Formation of primary neurons and formation of cranial nerves are affected. Hence, Hoxc6 functions are not restricted to the expected role in anterior-posterior pattern formation, but they are crucial for the initial formation and differentiation of primary neurons in the nervous system of *Xenopus laevis* as well.

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Introduction

Hox genes encode a family of transcription factors related to the homeotic factors of *Drosophila* (Akam 1989; Lemons and McGinnis 2006), where they were initially discovered (Lewis 1978). *Hox* genes are crucial for patterning and organizing structures along the main body axis (Krumlauf 1994). These genes are organized in clusters in a wide range of animals. Vertebrates possess multiple copies of *Hox* gene clusters, as a result of successive genome duplications (McGinnis and Krumlauf 1992; Lemons and McGinnis 2006; Duboule 2007; Deschamps 2007). During development, *Hox* genes are activated sequentially according to their position within the cluster, leading to unique combinations of *Hox* genes expressed at different axial levels, referred to as Hox code (Duboule and Morata 1994; Kessel 1994; Kessel and Gruss 1991). When ectopically expressed or mutated, *Hox* genes lead to so called homeotic transformations, i.e. one part of a body is transformed into another.

Hox gene functions have been extensively studied by generation of gain-of-function and loss-of-function mutants. This confirmed the role of *Hox* genes in defining axial identities (McGinnis and Krumlauf 1992). Furthermore, *Hox* genes were also connected to the patterning of the neural tube and its subtypes of neurons (Dasen et al. 2003; Song and Pfaff 2005; Nordström et al. 2006; di Sanguinetto et al. 2008). *Hox* gene expression has been correlated to the identities of motor neurons along the spinal cord (Shah et al. 2004). Indeed, in the developing spinal cord, motor neurons occupy discrete columns with different identities and projections (Tanabe and Jessell 1996). This columnar organization has been proven to be under the control of sequential phases of different Hox-C protein activities, in response to some gradient molecules, like the fibroblast growth factors (Dasen et al. 2003; Liu et al. 2001; Bel-Vialar et al. 2002; Guthrie 2004).

Hoxc6, an *Antennapedia* homologue, was the first *Hox* gene cloned in the vertebrate *Xenopus* (Carrasco et al. 1984). Two different proteins were described, a long and a short protein, due to the existence of two distinct promoters (PRI and PRII respectively, (Cho et al. 1988)). The two proteins share an identical DNA binding sequence, the homeodomain. The localization of these Hoxc6 proteins has been reported in previous studies.

The generation of a polyclonal anti-serum against Hoxc6 (formerly called XlHbox1) proteins enabled immuno-localization studies in *Xenopus*, mouse and zebrafish on the basis of the sequence conservation between the different species (Oliver et al. 1988; Wright et al.

1989; Molven et al. 1990). These studies focused on late stages of development. Hoxc6 protein localization was analyzed in stage 12 and 13 mouse embryos, and in a stage 45 *Xenopus* tadpole (Oliver et al. 1988). Hoxc6 proteins were found in the nervous system. In particular they were found in some neuronal populations as sensory neurons and interneurons in zebrafish, and in dorsal root ganglia in *Xenopus laevis* (Cho et al. 1988; Molven et al. 1990). Another study was based upon Northern-blot data on a stage 41 tadpole, and showed *in situ* hybridization on sections of a few additional stages using a probe recognizing both forms (at that time called *Xeb-1* (Carrasco and Malacinski 1987)). However, the analysis of expression of *Hoxc6* transcripts in early development has been quite incomplete, especially in relation to their potential functions with respect to primary neuron formation and patterning.

Here we show a detailed expression analysis of the two *Hoxc6* transcripts in *Xenopus laevis* during early development. We mainly focused on gastrulation and neurulation, using whole mount *in situ* hybridization for both transcripts. They exhibit different spatial patterns of expression, whereas their temporal patterns look identical. The two transcripts are predominantly co-expressed in the spinal cord during development of *Xenopus laevis* in accordance with the previous immuno-localization data.

We analyzed the functions of the two transcripts by loss-of-function and gain-of-function experiments. Specific depletion of either one of the two transcripts failed to affect pan-neural markers, whereas, after the double knockdown, the expression of *N-CAM* was reduced. However, the formation of primary neurons was affected by a single knockdown of either one of the isoforms. This neuronal phenotype is reversed by ectopic expression of *Hoxc6*. The results of our experiments point towards a novel basic function of *Hoxc6*, not in anterior-posterior patterning, but more generally in the formation and differentiation of primary neurons *in Xenopus laevis*.

Results and Discussion

Temporal and spatial expression patterns of two *Hoxc6* transcripts

The *Xenopus Hox* gene *Hoxc6* is under the control of two promoters, PRI and PRII (Cho et al. 1988). The organization of the two transcripts in *Xenopus laevis* is depicted in Figure 1

A. The distal PRI promoter is a common 5‘ regulatory unit for the *Hoxc4*, *Hoxc5*, and *Hoxc6* genes, whereas the proximal promoter PRII exclusively regulates *Hoxc6* (Simeone et al. 1988; Boncinelli et al. 1991; Coletta et al. 1991). The PRI promoter leads to the production of a transcript of 2.2 Kb. This long transcript encodes the shorter protein of 152 amino acids (hereafter called short form, SF). The PRII promoter leads to a smaller transcript of 1.8 Kb and encodes a longer protein of 234 amino acids (hereafter called long form, LF). The two proteins differ only in 82 amino acids at their N-terminus, but share an identical C-terminus including the homeodomain (Cho et al. 1988; Chariot and Gielen 1998; Sharpe et al. 1988; Shimeld et al. 1993).

The different localizations of these Hoxc6 proteins from the aforementioned immunohistochemistry in *Xenopus*, mouse and zebrafish, lead to the conclusion that the *Hoxc6* gene might be under tissue specific regulation (Oliver et al. 1988; Wright et al. 1989; Molven et al. 1990). The localization of transcripts in the whole embryo during the early development of mouse, frog or fish has only partially been documented. Here we have analyzed the temporal and spatial expression patterns of the *Hoxc6* isoforms in *Xenopus laevis* by RT-PCR and whole mount *in situ* hybridization, from the one cell stage until tadpole stages. In Figure 1 A, the positions of the PCR primers are indicated. These were also used to generate the probes for the *in situ* hybridization.

Both, the *Hoxc6* LF and SF transcripts are maternally expressed, although the level of LF is very low. Their maternal expression decreases rapidly (Figure 1 B). Zygotic expression appears during mid-gastrulation (after stage 11). Their expression persists during gastrulation and neurulation. Both transcripts have constant levels of expression at least until stage 27 (Figure 1 B).

The spatial expression patterns of both *Hoxc6* transcripts were analyzed by whole mount *in situ* hybridization. The probe recognizing the SF transcript is specific for this transcript. The probe for the LF transcript is located in the ORF and spans the first 246 nucleotides specific to the LF and 210 nucleotides common to both isoforms.

In accordance with the temporal expression patterns, neither transcript was detected by *in situ* hybridization at early gastrulation (Figure 1, C and L). Expression of both isoforms was found from midgastrula stages until tadpole stages. The expression increases during gastrulation. At the end of gastrulation, both transcripts were found in the posterior axial and paraxial regions (Figure 1 D, E and M, N). As neurulation proceeds, both isoforms were prominently expressed in the neural tube region, in the paraxial mesoderm and in the tailbud (Figure 1, F-H and O-Q). At tadpole stages, the expression of both transcripts persists mainly in the neural tube (Figure 1 I-K and R-T). The LF transcript shows a sharp

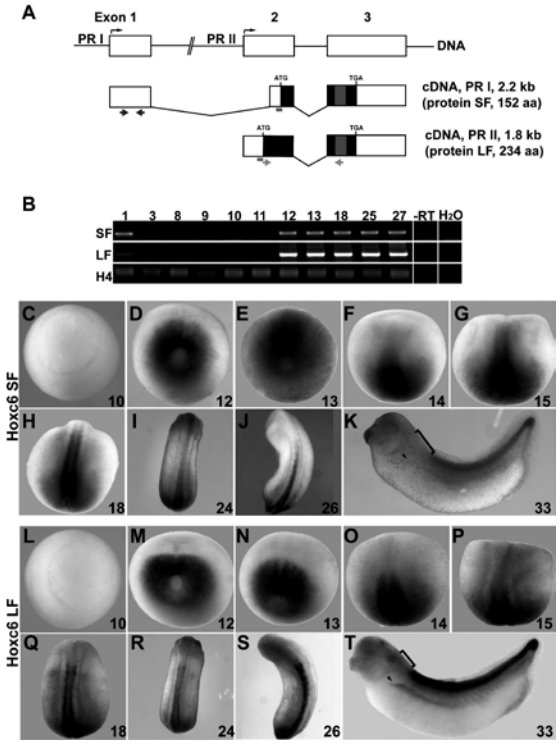


Figure 1. Temporal and spatial expression pattern of the *Hoxc6* isoforms

A Structure of *Hoxc6* isoforms in *Xenopus laevis*. *Hoxc6* isoforms derive from 2 different promoters. The distal promoter (PR I) leads to a 2.2 Kb precursor transcript, coding for the short form (SF) protein of 152 aa. The proximal promoter encodes a 1.8 Kb transcript leading to the long form (LF) protein of 234 aa. Black boxes indicate the open reading frame, grey boxes are the homeodomain. Red bars indicate the binding sites of the morpholino-oligonucleotides. The blue and green arrows show the primer sites that served for the RT-PCR and to generate the probes for *in situ* hybridization.**B** Temporal expression pattern of *Hoxc6* isoforms. RT-PCR was carried out using embryos at the indicated stages from 1 to 27. *H4* is used as a loading control, –RT is shown for stage 12 RNA.**C to K** show SF expression patterns from beginning of gastrulation until tadpole stages. Stages are indicated by the numbers **L to T** show the LF pattern from beginning of gastrulation until tadpole stages. The arrowheads in **K** and **T** indicate the location of the pronephric anlage. The squared brackets indicate the different distances between the anteriormost border of expression of each isoform and the otic vesicle area.

anterior boundary of expression in the neural tube. The transcript encoding the SF was detected with a more caudally located anterior boundary in the neural tube (using the otic vesicle as a landmark). Its pattern is more diffuse than that of the LF transcript (Fig.1, J-K and S-T, squared brackets). Moreover, at tadpole stages, expression of the LF transcript was found in the region of the pronephric anlage, while SF transcripts were not detected in this region (Fig.1, K, T, arrowheads). Our data show that the two *Hoxc6* transcripts do not have identical expression patterns. The anterior expression border of the SF appears to be more posterior. In addition the SF is not expressed in the pronephros or pronephric duct. To confirm the tissue localization of *Hoxc6* transcripts, we performed transversal sections of tadpoles. The *Hoxc6* expression was compared to those of known tissue specific markers. Thus, comparable sections were stained for *MyoD*, a somite specific marker (Hopwood et al. 1989, Figure 2 E), for *Xlim-1*, labeling the intermediate mesoderm of the pronephric anlage and the neural tube (Chan et al. 2000, Figure 2 F), and for *FoxF1* as a marker for lateral plate mesoderm (Köster et al. 1999, Figure 2 G).

Strongest expression for both isoforms of *Hoxc6* was found in the neural tube with exception of the floor plate (Figure 2 A-D). Comparing the expression of the *Hoxc6* isoforms to the other markers confirmed that both *Hoxc6* transcripts are expressed in somitic tissue. An additional expression domain of the LF was found in the pronephric anlage. The schemes in Figure 2 H indicate the position of the sections within the embryo, and the positions of the different tissues within the sections. In addition sections were analyzed originating from regions in front of the expression domain, as detected by whole mount *in situ* hybridization. Interestingly expression of both, the LF and the SF, were detected in the neural tube at the level of branchial arches (Figure 2 I, J).

These observations highlight the difference in tissue expression between the two *Hoxc6* transcripts. Both forms are expressed within the neural tube, and in the somitic mesoderm. However, staining in the cross sections also demonstrates a specific high level of expression of the LF transcript in the pronephric anlage. These spatial data suggest that the expression of *Hoxc6* transcripts is differentially regulated in different tissues.

Knockdown of either one of the isoforms of *Hoxc6* failed to affect *N-CAM* and *Notch* expression in the neural plate

To investigate *a* possible function of *Hoxc6* during *Xenopus* neurulation, we performed loss-of-function studies using antisense morpholino-oligonucleotides against both *Hoxc6* isoforms (MO; their positions are indicated as red bars in Figure 1 A). First, the function of *Hoxc6* MOs was tested *in vitro* in a transcription-translation assay to verify that *Hoxc6* transcripts are inhibited efficiently (Figure 3 A). MO2 preferentially blocks translation of the SF, and MO3 exclusively inhibits the translation of the LF. This nomenclature for the MOs will be used in the description below. The addition of the standard control MO (ctMO) does not affect the efficiency of translation in the reticulocyte lysate of either of the wild type *Hoxc6* forms. A construct coding for a LF transcript lacking the MO3 binding site (Figure 3 A, LF(insens.)) was tested in this *in vitro* assay as well. MO2 does recognize an internal sequence within the LF transcript. To exclude strong cross reactions we tested the potential interference of MO2 with the translation of the LF(insens.) construct. We found that addition of either MO2 or both, MO2 and MO3, failed to block the translation of this insensitive transcript of the LF (Figure 3 A). This confirms that MO2 does not efficiently disturb the translation of the LF transcript, and therefore proves its specificity to knockdown the SF.

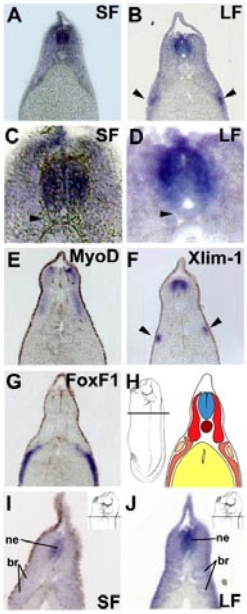


Figure 2. Tissue localisation of *Hoxc6* isoforms in cross sections

A SF expression in the somitic tissue and neural tube. **B** Expression of LF in somitic tissue and neural tube; in addition LF is expressed in the pronephric anlage (arrowheads). **C, D** Close-ups of the neural tubes shown in **A** and **B**. Expression of the *Hoxc6* isoforms is detected in all cells of the neural tube with exception of the floorplate. **E** *MyoD* expression in the somites. **F** *Xlim-1* expression in the neural tube and in the pronephric anlage (arrowheads). **G** *FoxF1* expression in the lateral plate mesodem. **H** Position of the shown sections within stage 27 embryos, and localization of different tissues in the sections. Neural tube (blue); somite mesoderm (red); notochord (brown), endoderm (yellow), lateral plate mesoderm (dark orange), the pronephric anlage (light orange). **I** Cross section at the level of the branchial arches (br) as indicated in the inlay. Expression of SF is found in the neural tube (ne), even though it was not visible in whole mount *in situ* hybridization. **J** Expression of LF in the neural tube at the same level.

To analyze the endogenous function of *Hoxc6* transcripts for neural induction as the first step of neurogenesis, MO2 or MO3 were injected at the 2-cell stage into both cells at the animal pole of embryos. We monitored the expression of the neural marker *Sox-2*, which labels the neural plate in the *Xenopus* neurula (Mizuseki et al. 1998). Neither injection of MO2, knocking down the SF (Figure 3 D), nor injection of MO3, knocking down LF (Figure 3E), nor the combined injection of both MOs (Figure 3 F) showed differences compared to uninjected or ctMO injected embryos (Figure 3 B, C). Identical results were obtained for another two neural markers (*Nrp-1*, *Xenopus Notch*, shown in the Supplemental Figure 1 A-J). It has been reported that Hoxc6 proteins bind to the promoter of the general neural marker *N-CAM* and activate its transcription in NIH 3T3 cells. This activation is stronger, when both isoforms bind the promoter simultaneously (Jones et al. 1993).

Thus, we asked whether depletions of *Hoxc6* transcripts have any effect on *N-CAM* expression *in vivo*. *N-CAM* expression was not affected by ctMo injection compared to uninjected controls (Figure 3 G, H). After depletion of either one or the other transcript, *N-CAM* expression was not significantly affected (Figure 3 I, J). Nevertheless, combined injection of the two MOs led to a dramatic down-regulation of *N-CAM* expression (Figure 3 K). An identical effect was observed on the epidermal ectoderm marker *XK81A1* (Supplemental figure 1 K-O).

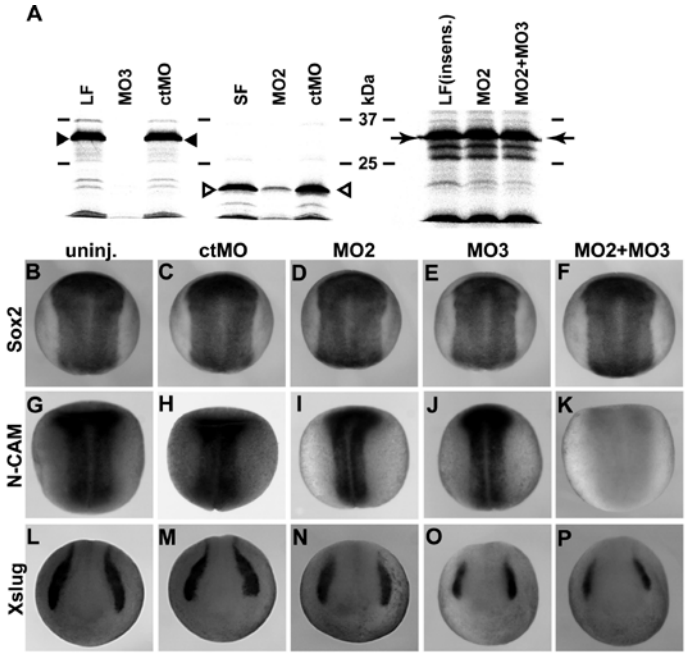
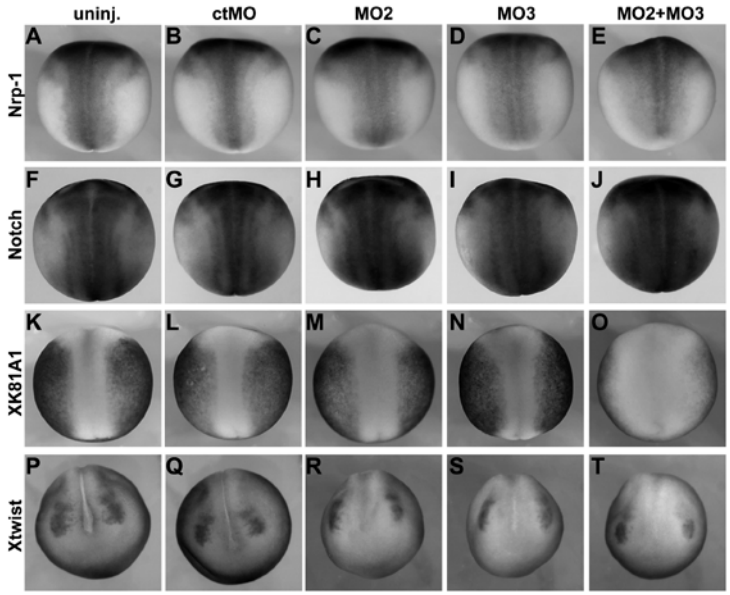


Figure 3. The effect of *Hoxc6* knockdowns on neural markers

A Specificity of MO nucleotides was tested *in vitro* using a coupled transcription-translation assay. *In vitro* synthesis of LF was inhibited by MO3, but not by the ctMO (black arrowheads). *In vitro* synthesis of the SF was reduced by MO2, but not by ctMO (open arrowheads). Translation of a LF construct lacking the MO3 binding site (LF insensitive) is neither inhibited by MO2 nor by MO2 combined with MO3 (arrows). This demonstrates that the SF specific MO2 does not affect synthesis of the LF, even though its target sequence is present in the LF mRNA.**B to F** The neural marker *Sox2*

The analysis of neural crest markers revealed a significant effect of the knockdown of the LF (separate or combined with the knockdown of SF), but weak or no effects of the knockdown of the SF (*Xslug* in Figure 3 L-P, *Xtwist* in Supplemental Figure 1 P-T). The effects of a knockdown of *Hoxc6* on the epidermal and neural crest ectoderm are unexpected, since no expression of Hoxc6 was detected in these tissues at the analyzed stages. We do not have an explanation yet. One can speculate that is effect must be indirect, acting via the inhibition of Hoxc6 in the neuroectoderm or the mesoderm.

Altogether, our data indicate that depletion of a single *Hoxc6* isoform in *Xenopus* does not affect general neural induction or the initial neural tissue formation. We also show that *N-CAM* expression in the *Xenopus* embryo is under a synergetic control of both *Hoxc6* products as has previously been reported for a cell culture (Jones et al. 1993).



Supplemental Figure 1. The effect of *Hoxc6* knockdowns on different markers

Expression patterns of different markers in uninjected embryos, and after the injection of ctMO, MO2, MO3, and a combination of MO2 and MO3 as indicated above the photographs. **A to E** Expression of the neural marker *Nrp-1*. **F to J** Expression of the neural marker *Notch*. **K to O** Expression of the epidermal marker *XK81A1*. **P to T** Expression of the neural crest marker *Xtwist*.

Knockdown of either one of the isoforms of *Hoxc6* leads to defects in formation of primary neurons

Neural development starts with neural induction during gastrulation. A few hours later the first neurons begin to differentiate in the *Xenopus* neural plate, located in three bilateral longitudinal stripes, so called columns. This pattern reflects the functional subdivision of the early nervous system. Thus, motor neurons, interneurons and sensory neurons derive from the medial, intermediate and lateral column respectively (Hartenstein 1993; Diez del Corral and Storey 2001). It has been shown that Hox-C proteins can influence the identity of motor neurons within the spinal cord columns of chicken (Dasen et al. 2003; Dasen et al. 2005).

Furthermore, *Hox* mutants have been shown to exhibit defects in motor axon projections, consistent with an alteration in their identity (Wahba et al. 2001; Tiret et al. 1998; Wu et al. 2008). We therefore asked whether a *Hoxc6* knockdown has any influence on the formation and identity of primary neurons in *Xenopus*. We monitored the expression of *N-tubulin* upon MO injections. *N-tubulin* is a pan-neuronal marker expressed in neurons undergoing the differentiation process (Figure 4 A). Its expression is not affected by the injection of the ctMO (Figure 4 B). Injection of MO2 resulted in, at most, weak effects (Figure 4 C). When the MO3 was injected, *N-tubulin* expression was extensively down-regulated (Figure 4 D), indicating an impaired formation of primary neurons. Half-sided injections of the different MOs (Supplemental Figure 2 A-D) and the comparison with ctMO injections show that this down-regulation does not result from a delay in development. The knockdown of the LF by injection of MO3 was counteracted by co-injection of the insensitive LF-mRNA, which in addition resulted in ectopic *N-tubulin* expression (Figure 4 E). It is worthwhile noticing that the loss of the LF transcript leads to a stronger effect than the depletion of the SF transcript. This could indicate a potential difference in the functions of the corresponding Hoxc6 proteins. Next we investigated the effect of Hoxc6 depletion on some subpopulations of neurons. The LIM homeodomain protein Xisl-1 is expressed in the medial and lateral parts of the spinal cord. Amongst others it controls the trajectory of the motor axons along the dorso-ventral axis of the limb (Kania and Jessell 2003).

In an uninjected or a ctMO injected neurula stage embryo, *Xisl-1* positive neurons form a discrete set of columns reminiscent of *N-tubulin* expression pattern, but with fewer cells (Figure 4 F, G). The depletion of either the SF transcript or the LF transcript causes a decrease in number of *Xisl-1* positive cells (Figure 4 H, I, Supplemental Figure 2 E-G). Again, the loss of the LF transcript gives a severe phenotype and the depletion of the SF a weak effect. Next we investigated the effect of Hoxc6 depletion on some subpopulations of neurons. The LIM homeodomain protein Xisl-1 is expressed in the medial and lateral parts of the spinal cord. Amongst others it controls the trajectory of the motor axons along the dorso-ventral axis of the limb (Kania and Jessell 2003). In an uninjected or a ctMO injected neurula stage embryo, *Xisl-1* positive neurons form a discrete set of columns reminiscent of *N-tubulin* expression pattern, but with fewer cells (Figure 4 F, G). The depletion of either the SF transcript or the LF transcript causes a decrease in number of *Xisl-1* positive cells (Figure 4 H, I, Supplemental Figure 2 E-G). Again, the loss of the LF transcript gives a severe phenotype and the depletion of the SF a weak effect. Later on, *Xisl-1* is expressed in cranial nerves, in the neural crest of the branchial arches, and along the spinal cord as described ((Brade et al. 2007), and Figure 4 K, L).

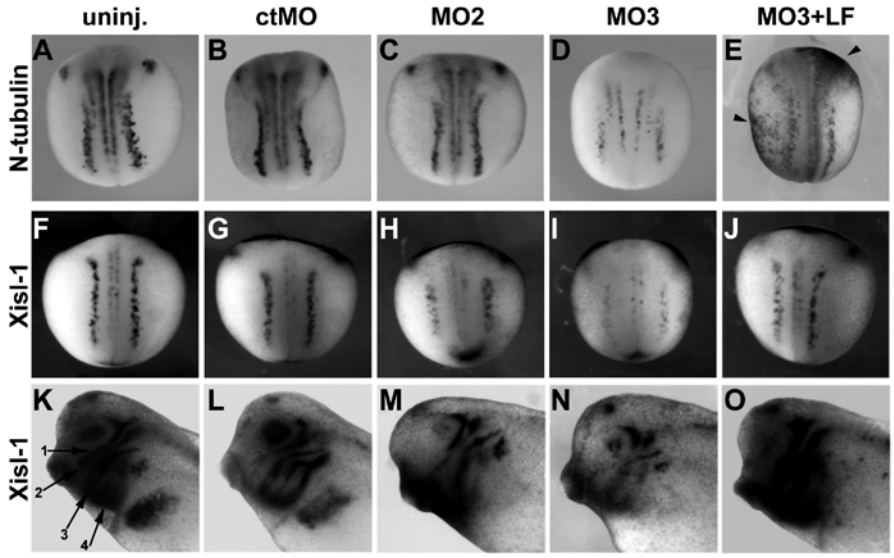
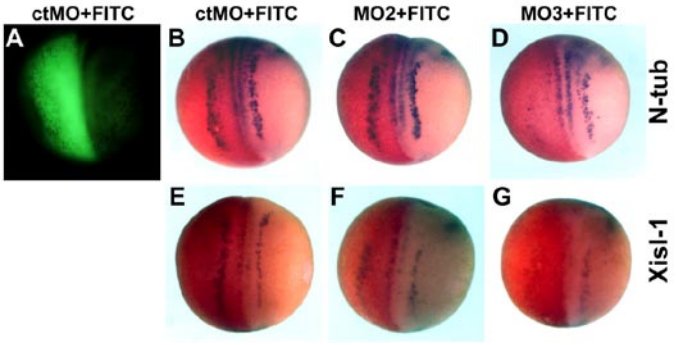


Figure 4. The effect of *Hoxc6* knockdowns on formation of primary neurons and cranial nerves

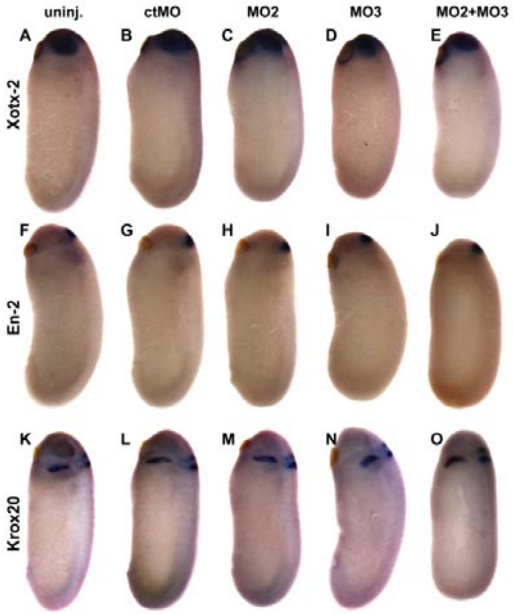
Markers specific for primary neurons (*N-tubulin*, *Xisl-1*) and for cranial nerves (*Xisl-1*) were analyzed at a neurula stage and a tadpole stage by *in situ* hybridization upon *Hoxc6* depletion. Shown are uninjected (uninj.) embryos, and embryos injected with ctMO, MO2, MO3, and a combination of MO3 with an MO-insensitive mRNA for LF, as indicated above the photographs.**A to E** Analysis of the neuronal marker *N-tubulin*. Arrowheads indicate ectopic N-tubulin expression.**F to J** Expression of *Xisl-1* at a neurula stage for the same set of treatments. No ectopic expression was found.**K to O** Expression of *Xisl-1* in the head of a tadpole. Numbers in **K** indicate the different branchial arches and the corresponding cranial nerves.

Especially the *Hoxc6* LF hypomorph shows a severe decrease in *Xisl-1* expression in the corresponding structures (Figure 4 M, N). These results are in accordance with previous studies. With *in vivo* injections of a functional antibody it has been shown that inhibition of the function of the LF protein leads to severe defects at the level of hindbrain and anterior spinal cord (Wright et al. 1989). Moreover, in a rescue experiment we find that co-injection of MO3 with an MO- insensitive LF transcript restores the loss of *Xisl-1* positives neurons (Figure 4 J) and of *Xisl-1* expression in the region of the cranial nerves (Figure 4 O). However, in contrast to the *N-tubulin* expression no significant ectopic activation was observed for Xisl-1. This rescue experiment highlights a predominant role of *Hoxc6* (at least the LF protein) in regulating *Xisl-1* positive neurons in *Xenopus*, as it does in chicken (Dasen et al. 2005).



Supplemental figure 2 Half-sided knockdowns of the *Hoxc6* isoforms confirm an *Hoxc6*-specific effect on neuronal markers

Embryos were injected in one out of two blastomeres with a mix of FITC-dextran and the different MOs. The different combinations are indicated above the photographs.**A** In vivo photograph of an half-sided injected embryo. **B to D** *N-tubulin* expression after half-sided injections. The Fast-Red staining of FITC indicates the injected side. **E to G** Expression of *Xisl-1* after half-sided injections. Again the Fast-Red staining of FITC indicates the injected side.



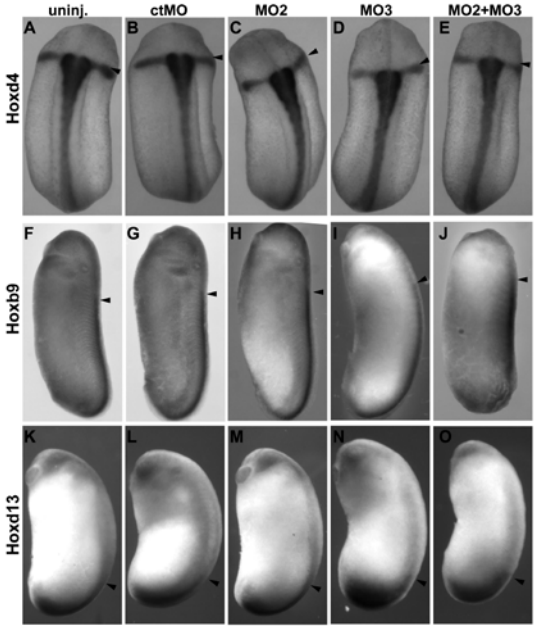
Supplemental figure 3. The effect of *Hoxc6* knockdowns on anterior-posterior marker genes of the head region

A to E Expression of the anterior marker *Xotx-2* in uninjected embryos, and in embryos injected with ctMO, MO2, MO3, or with the combination of MO2 and MO3, as indicated above the photographs. **F to J** Expression of the midbrain-hindbrain boundary marker *En-2* after the same set of treatments. **K to O** Expression of the hindbrain marker *Krox-20* after the same set of treatments.

Interestingly, at least parts of this effect must be indirect, since it affects cells (i.e. the cranial nerves), which do not express *Hoxc6* at the analyzed stages. We could not confirm the idea that the effect may result from a homeotic transformation, since the analysis of a series of anterior-posterior marker genes did not reveal significant changes in the anterior-posterior patterning (Supplemental Figure 3 and 4). The observed (and probably indirect) effect on early neural crest formation may be part of the explanation (see above).

Together, our results show that the depletion of Hoxc6 proteins leads to a general loss in primary neuron formation as shown by a down-regulation of *N-tubulin*, and especially a loss of *Xisl-1* positive neurons.

As indicated above, this effect is neither due to a reduction of neural precursors, nor the result of changes in anterior-posterior patterning. Rather, the quantity or quality of differentiation of primary neurons from these cells is affected.



Supplemental figure 4. The effect of *Hoxc6* knockdowns on anterior-posterior marker genes of the trunk region

A to E Expression of *Hoxd4* in uninjected embryos, and in embryos injected with ctMO, MO2, MO3, or with the combination of MO2 and MO3, as indicated above the photographs. **F to J** Expression of *Hoxb9* after the same set of treatments. **K to O** Expression of *Hoxd13* after the same set of treatments. Arrowheads in all photographs indicate the anterior border of expression seen in whole mount *in situ* hybridization.

This decrease in the number of *Xisl-1* positive neurons was reversed by co-injection of the MO3 (knocking down the *Hoxc6* LF) with the mRNA encoding a MO-insensitive form of *Hoxc6* LF. These results highlight a major function of *Hoxc6* in neurogenesis in *Xenopus* laevis. In contrast to neural induction, formation and differentiation of primary neurons is strongly affected by the depletion of the *Hoxc6* LF product indicating a crucial role of *Hoxc6* in the formation and differentiation of primary neurons. This places Hoxc6, on the pathway of neurogenesis, between neural induction and the specification of primary neurons.

Injection of mRNAs encoding either one of the isoforms of *Hoxc6* leads to ectopic expression of the neuronal marker *N-tubulin*

In the rescue experiment shown in Figure 4 we observed ectopic expression of the neuronal marker *N-tubulin*. We therefore asked, if the injection of mRNA encoding either one of the *Hoxc6* isoforms ectopically activates neural and neuronal markers. In comparison to uninjected embryos the expression of the general neural marker *N- CAM* was not significantly affected after injections of LF or SF mRNA (Figure 5 A-C). The neuronal marker N-tubulin is ectopically activated in both, embryos injected with LF mRNA and embryos injected with SF mRNA (Figure 5 D-F). Surprisingly no ectopic expression of *Xisl-1* was found in corresponding embryos of the same experiments. The explanation may be that the Hoxc6 ectopically induces primary neurons different from the *Xisl-1*-positive motor neurons. Further experiments confirm a close relation of *Hoxc6* to *N-tubulin*. A comparison of the temporal and spatial expression patterns of *N-tubulin* with *Hoxc6* patterns shows extensive overlaps. Expression of *N-tubulin* was detected from midgastrula stages on, overlapping with *Hoxc6* in time (Figure 6 A, Figure 1 B) and space (Figure 6 C-F, spatial Hoxc6 patterns in Figure 1). We also analyzed the effect of ectopic expression of the *Hoxc6* isoforms on animalcaps at a neurula stage. RT-PCR data show that injection of either SF mRNA or LF mRNA resulted in activation of *N-tubulin* expression (Figure 6 B). In accordance with our gain-of-function experiments, *N-CAM* was not activated in these caps. As a positive control injection of the mRNA for the neural inducer Noggin was used. In these *noggin* injected caps both, *N-CAM* and *N-tubulin*, are activated. The activation of *N-tubulin* by *Hoxc6* injection appears already during gastrulation, as it is shown by the half-sided injection of the LF mRNA (Figure 6 G). Rescue with mRNAs for both, LF and SF, on MO2 or MO3 injected embryos confirm this role of *Hoxc6* for the regulation of

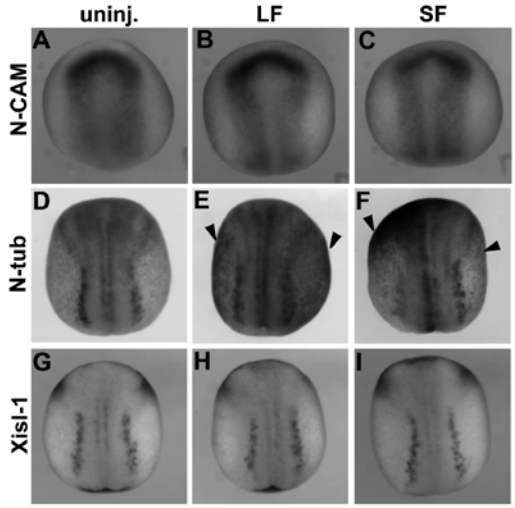


Figure 5 Effect of overexpression of the LF and the SF on neural and neuronal markers.

A to C Expresssion of the neural marker *N-CAM* in uninjected embryos (uninj.), and in embryos injected with mRNA encoding either the LF or the SF as indicated above the photographs.**D to F** Expression of the neuronal marker *N-tubulin* after the same set of treatments. Arrowheads indicate ectopic activation of *N-tubulin* expression.**G to I** Expression of the neuronal marker *Xisl-1* after the same set of treatments.

N-tubulin. Consistent with the experiments shown in figure 4, MO2 has a weak downregulating effect and MO3 has a strong downregulating effect on *N-tubulin* (Figure 7 A, F). Coinjection with different doses of LF mRNA did not result in a rescue after loss of *N-tubulin* with MO2 (Figure 7 B, C), whereas the MO3 effects were clearly rescued (Figure 7 G, H). In addition ectopic activation of *N-tubulin* was observed very often. Coinjections of the MOs with different doses of the SF mRNA rescued the weak MO2 effects (Figure 7 D, E). In MO3 injected embryos, we never observed restauration of *N-tubulin* even at such high doses of SF mRNA, that gastrulation is affected (Figure 7 I, J). Also ectopic *N-tubulin* activation is only infrequently found, in the shown cases presumably due to the malformations. So, in correspondence with the loss-of-function experiments, the gain-of-function experiments and the rescue experiments especially with *Hoxc6* LF show effects on the primary neuron formation, but not on the general neural marker *N-CAM*. This again places *Hoxc6* as a necessary component on the pathway of neurogenesis, in a position somewhere between the early steps of neural induction and the specification and differentiation of primary neurons.

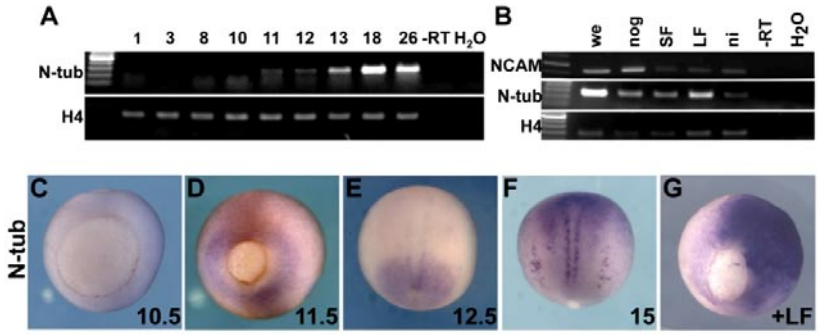


Figure 6 The early temporal and spatial pattern of *N-tubulin* and its activation by *Hoxc6* isoforms.

A A time course of *N-tubulin* expression analyzed by RT-PCR. Numbers indicate the stages. *H4* is used as loading control. **B** Activation of *N-tubulin* in animal caps by *Noggin* (nog), SF and LF at early neurulation. As additional controls uninjected animal caps (ni) and whole embryos (we) are shown. *H4* is used as loading control. **C to F** Spatial expression pattern of *N-tubulin* at gastrula and early neurula stages as indicated by the numbers. **G** Ectopic activation of *N-tubulin* during gastrulation after half-sided injection of LF mRNA.

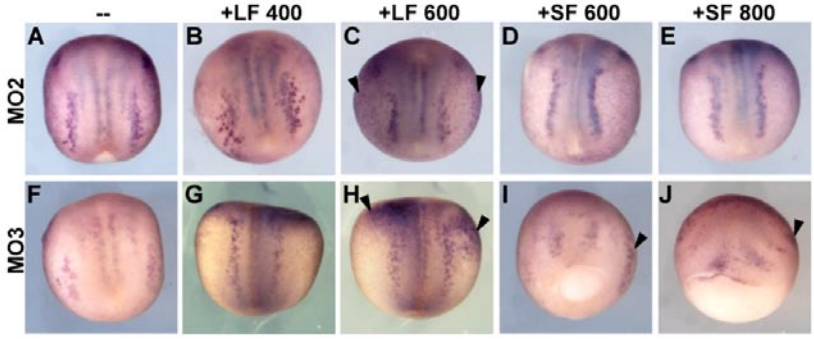


Figure 7 Effects of LF and SF mRNA on embryos either injected with MO2 or with MO3.

A to E Expression of *N-tubulin* is shown in embryos after injection with MO2 alone, and in combination with different doses of either LF or SF mRNA, as indicated above the photographs. The numbers describe the amount of mRNA in pg per embryo. Arrowheads indicate ectopic activation of *N-tubulin* expression. **F to J** Expression of *N-tubulin* in embryos after injection with MO3 alone and in combination with different doses of either LF or SF mRNA. Arrowheads in **G** indicate ectopic activation of *N-tubulin* expression. Expression labeled with arrowheads in **I** and **J** may be ectopic or may result from the defect of gastrulation.

Experimental Procedures

Cloning of the *Hoxc6* expression construct

The complete open reading frame of *Hoxc6* (BC084319) was amplified using primers including *BamHI* and *XhoI* restriction sites (F: 5'-**GGATCC**ATGAATTCCTATTTCACTAACCCTT-3'; R: 5'-**CTCGAG**GGGTGTCTCTCCATTCACTCTTT-3'). The short form *Hoxc6* was amplified using the following primers with *BamHI* and *EcoRI* restriction sites: (F: 5'-CG**GGATCC**ATGCTCACTAGCTGCAGGCAGA-3'; R: 5'-G**GAATTC**TCACTCTTTGCCTTGTCCCTCT-3'). After ligation of the PCR product into pGEM-T Easy vector (Promega), *Hoxc6* long form (LF, previously called PRII) was excised by *BamHI*/*XhoI* digestion and ligated into CS2+ vector (Turner and Weintraub 1994). The *Hoxc6* short form (SF, previously called PRI) was excised by EcoRI/BamHI and ligated to CS2+. These constructs were checked by sequencing. Since the LF and the SF constructs do not encode the 3' sequences that are recognized by the MOs (see below), they are insensitive to the MO knockdown.

Injection of morpholinos and mRNA

Embryos were staged according to (Nieuwkoop and Faber 1956). *In vitro* fertilization, embryo culture, and mRNA injection, were carried out as previously described (Wacker et al. 2000; Winklbauer 1990). Morpholino injections were done as mRNA injections, except that 5 nl of MO solution per cell were injected in both blastomeres at stage 2. The concentrations are stated below.

Two anti-sense morpholino oligonucleotides (MOs, Gene-Tools Inc.) were designed for the *Hoxc6* transcripts, which encode the two different isoforms. Each MO was designed to be effective against one of the isoforms (compare Figure 1 A). The sequences of the MOs are as follows: *Hoxc6*, MO2-5'-TCTATTACAACACAAACCGGAGGTCG-3', MO3-5'-ATTCATATCTTCTCCTTTACCTGCC-3'. MO3 targets the LF transcript, while MO2 is effective against the SF transcript. SF and LF are designating the size of the protein in the present report. Thus LF transcript refers to the formerly called PRII product. Respectively, SF refers to PRI. Morpholinos and mRNAs were diluted in Gurdon's buffer (15 mM Tris pH 7.5, 88 mM NaCl, 1 mM KCl) and injected at two cell stage in the animal hemisphere. The amounts injected are 15 ng or 20 ng per embryo of *Hoxc6* MOs and of the standard control MO. For some experiments MOs were co-injected with FITC dextran (M=10000, Molecular probes) in one cell out of two cell stage embryo. In all experiments, control MO (standard control, Gene-Tools Inc.) injections were also carried out and embryos processed for marker analysis. Control MO (ctMO) embryos never gave different results from the

uninjected controls. The function and specificity of the MOs were tested *in vitro* using the Sp6 coupled transcription and translation assay (Promega). For gain-of-function and rescue experiments 600 pg of *Hoxc6* MO-insensitive mRNA were used, if not differently stated. Up to 800pg of *Hoxc6* SF mRNA were injected alone or in combination with MOs.

Detection of gene expression by *in situ* hybridization

Whole mount *in situ* hybridization was performed as previously described (Harland 1991; Wacker et al. 2004), except that the RNAse step was omitted. Antisense digoxigenin-labeled probes were: *FoxF1* (*XFD13*, (Köster et al. 1999)), *Xlim1* (Chan et al. 2000), *N-tubulin* (Chitnis and Kintner 1995; Marcus et al. 1998), *Xisl-1* (Brade et al. 2007), *Sox-2* and *Hoxd4* (Jansen et al. 2007), *Hoxb9* and *Hoxd13* (Wacker et al. 2004), *MyoD* and *Xtwist* (Hopwood et al. 1989), *Krox-20* (Bradley et al. 1993), *En-2* (Hemmati-Brivanlou et al. 1991), *Xotx2* (Blitz and Cho 1995), epidermal keratin *XK81A1* (Jonas et al. 1989), *Nrp-1* (Richter et al. 1990), *Xenopus Notch* (Coffman et al. 1990), *Xslug* (Mayor et al. 1995), *N-CAM* (Kintner and Melton 1987). The SF probe was generated by cloning a part of the precursor messenger (Figure 1 A). The primers used are as follows: F 5'-GGAATTCGGAAAAGGCATTTGCACACGCCA-3'; R: 5'-CCGCTCGAGTGCTACAGCCCGGAACTCTGG-3'. The LF probe was cloned by amplifying a fragment from the start codon until the homeodomain from the plasmid coding for the long form protein. The primers used are: F 5'-ATGAATTCCTATTTCACTAACCCT-3'; R: 5'-TTTGGTAACGGGAATAGATCT-3'. The fragment was ligated to the *pDRIVE* vector. For embryos injected with MOs and FITC, A antiFITC-antibody and Fast-Red were used to reveal the FITC at first. The DIG labeled probes for *N-tubulin* or *Xisl-1* and their detection followed using the standard methods as mentioned above. In some cases embryos were bleached with hydrogen peroxide after the *in situ* hybridization. For sections embryos were embedded in gelatin blocks after *in situ* hybridization, and dissected at 50 µm intervals.

Animal cap assay

Embryos were injected at two cell stage in both cells with *noggin* (500 pg per embryo), *Hoxc6 LF* (600 pg per embryo), *Hoxc6 SF* (600 pg per embryo) mRNAs. Caps were dissected at stage 8.5 and cultured until control siblings reached stage 15. Samples were processed to RNA extraction and RT-PCR.

RNA isolation and RT-PCR analysis

Total RNA was extracted from different stages embryos or caps by Trizol (Invitrogen) and phenol chloroform extraction. The extract was cleaned using the RNAeasy columns kit (Qiagen). The first strand cDNA synthesis (Fermentas) was carried out according to the manufacturer instructions. The following primers were used:

H4 (F: 5'-CGGGATAACATTCAGGGTA-3'; R: 5'-TCCATGGCGGTAACTGTC-3'); SF (F: 5'-GGAATTCGGAAAAGGCATTTGCACACGCCA-3'; R: 5'-CCGCTCGAGTGCTACAGCCCGGAACTCTGG-3'; LF (F: 5'-ATGAATTCCTATTTCACTAACCCT-3'; R: 5'-TTTGGTAACGGGAATAGATCT-3'); N-tubulin (F: 5'-ACACGGCATTGATCCTACAG-3'; R: 5'-AGCTCCTTCGGTGTAATGAC-3'); N-CAM (F: 5'-GCCCCTCTTGTGGATCTTAGTGA-3'; R: 5'-ACAGCGGCAGGAGTAGCAGTTC-3').

The positions of the primers for LF and SF are indicated in figure 1 A.

Acknowledgements

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Chapter 4

Development 136 (2005) 2861-2867

Knockdown of the complete Hox paralogous group 1 leads to dramatic hindbrain and neural crest defects

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Summary

The Hox paralogous group 1 (PG1) genes are the first and initially most anterior Hox genes expressed in the embryo. In *Xenopus*, the three PG1 genes, *Hoxa1*, *Hoxb1* and *Hoxd1*, are expressed in a widely overlapping domain, which includes the region of the future hindbrain and its associated neural crest. We used morpholinos to achieve a complete knockdown of PG1 function. When *Hoxa1*, *Hoxb1* and *Hoxd1* are knocked down in combination, the hindbrain patterning phenotype is more severe than in the single or double knockdowns, indicating a degree of redundancy for these genes. In the triple PG1 knockdown embryos the hindbrain is reduced and lacks segmentation. The patterning of rhombomeres 2 to 7 is lost, with a concurrent posterior expansion of the rhombomere 1 marker, *Gbx2*. This effect could be via the downregulation of other Hox genes, as we show that PG1 function is necessary for the hindbrain expression of Hox genes from paralogous groups 2 to 4. Furthermore, in the absence of PG1 function, the cranial neural crest is correctly specified but does not migrate into the pharyngeal arches. Embryos with no active PG1 genes have defects in derivatives of the pharyngeal arches and, most strikingly, the gill cartilages are completely missing. These results show that the complete abrogation of PG1 function in *Xenopus* has a much wider scope of effect than would be predicted from the single and double PG1 knockouts in other organisms.

Key words: Hox, PG1, *Xenopus*, Hindbrain, Neural crest

Introduction

A vital aspect of vertebrate development is the organisation and patterning of different tissues along the various axes of the embryo. Along the anteroposterior (AP) axis this occurs partly under the control of the Hox family of transcription factors. These homeobox-containing genes have been conserved throughout evolution and are responsible for the patterning of various tissues at specific axial levels (McGinnis and Krumlauf, 1992). In vertebrates, the Hox genes are organised in multiple clusters, which contain 13 paralogous groups, identified on the basis of DNA sequence and the position on the chromosome. These clusters are believed to be derived from the successive duplications of an ancestral Hox cluster. The subsequent loss of certain genes, and divergence and function shuffling in the remaining genes resulted in the clusters as they are now (Prince and Pickett, 2002). One interesting aspect of the Hox genes is that they are expressed in a colinear fashion. This means that genes which are located more 3′ in the cluster are generally expressed at an earlier developmental time point [temporal colinearity (reviewed by Deschamps et al., 1999)] and also at a more anterior position in the embryo [spatial colinearity (reviewed by Duboule and Dolle, 1989; Graham et al., 1989)]. The mechanism behind this colinearity is not yet delineated but the final effect is to produce a complex pattern of Hox gene expression, known as the 'Hox code', which defines particular AP axial levels (Deschamps et al., 1999; Maconochie et al., 1996).

Often however, the removal of the function of one particular Hox gene, or even one complete cluster, does not have dramatic consequences for the embryo (Spitz et al., 2001; Suemori and Noguchi, 2000). This, together with the observation that paralogous genes often have similar functions as well as similar expression domains, points to the possibility of functional redundancy between genes from the same paralogous group (PG). This indeed appears to be the case. The loss of function of complete paralogous groups have been shown to be more severe than knockouts of single Hox genes in both zebrafish [PG2 (Hunter and Prince, 2002)] and mouse [PG8 (van den Akker et al., 2001)]. Thus the knockdown of a single Hox gene may not reveal its complete function, and entire paralogous groups may need to be abrogated before their shared role can be illuminated.

Here we investigate the function of the PG1 genes, which are the homologues of the *Drosophila labial* gene. These are the earliest of the Hox genes, with expression starting during gastrulation, and they eventually have their anterior boundary in the hindbrain at the level of rhombomeres (r) 3/4 in most vertebrates (Frohman et al., 1990; Frohman and Martin, 1992; Godsave et al., 1994; Kolm and Sive, 1995; Murphy and Hill, 1991; Sundin et al., 1990; Wilkinson et al., 1989). *Hoxa1* is also expressed at a later stage in the fore/midbrain (McClintock et al., 2002; Shih et al., 2001).

Knockout studies in mouse have concentrated on the *Hoxa1* and *-b1* genes, and have implicated these genes in hindbrain and craniofacial development. In the *Hoxa1* null mutant, r5 is either absent or severely reduced, r4 is reduced and there are

defects in the hindbrain and associated nerves in the region between r3 and r8 (Carpenter et al., 1993; Chisaka et al., 1992; Dolle et al., 1993; Lufkin et al., 1991; Mark et al., 1993). In the *Hoxb1* knockout the identity of r4 is altered, but segmentation is not affected (Goddard et al., 1996; Studer et al., 1996). When both *Hoxa1* and *Hoxb1* are deleted, a more severe phenotype is observed than in either of the single knockouts, with both r4 and r5 being mis-specified (Gavalas et al., 1998; Studer et al., 1998) and eventually deleted (Rossel and Capecchi, 1999) as well as the 2nd pharyngeal arch and its derived tissues being lost. In zebrafish, knockdown of the *Hoxb1b* gene (thought to be the functional equivalent of the mouse *Hoxa1* gene) leads to disruption of r4 and *Hoxb1a*, like mouse *Hoxb1*, is involved in the specification of nerves originating in r4. The double knockdown of *Hoxb1b* and *Hoxb1a* also implies some degree of functional redundancy between these genes. However, the phenotype of the double knockdown is not as severe as that observed in mouse, with r4 and r5 always present, albeit reduced (McClintock et al., 2002).

Despite the intense interest in these anterior Hox genes, a complete knockdown of all PG1 genes has not yet been performed. Therefore any function that is shared between all of the genes may still be hidden. To address this question we knocked down all the *Xenopus laevis* Hox PG1 genes. In *Xenopus*, the early expression of the three PG1 genes (*Hoxa1*, *Hoxb1* and *Hoxd1*) is highly overlapping and they are all expressed in the presumptive hindbrain region. Here we use the morpholino (MO) knockdown technique to demonstrate that the complete loss of PG1 gene function has deeper implications for the development of the embryo than the loss of the individual genes.

Materials and methods

Embryos and explants

Xenopus laevis embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). Culture of embryos and buffers was as described (Winklbauer, 1990).

Cloning of the *Xenopus Hoxd1* morpholino insensitive construct

The complete open reading frame of *Xenopus Hoxd1* (Sive and Cheng, 1991) was amplified using primers incorporating *Bam*HI and *Xho*I restriction sites (for: 5′CCGGGATCCGCCGCCACCATGAATTCCTACCTAGAATACACTTCTTGCGGG; rev: 5′TGCACTCGAGCTAGGGTGAAGCGTCCTTGGATGGCG). After ligation of the PCR product into the pGEM-T Easy vector (Promega), *Hoxd1* was excised by *Bam*HI/*Xho*I digestion, ligated into the CS2+ vector (Turner and Weintraub, 1994), and checked by sequencing.

Injection of morpholinos and mRNA

Two morpholinos were designed for each Hox PG1 gene (Gene-Tools Inc.) The pseudo-tetraploidy of *Xenopus laevis* was taken into consideration and the morpholinos were all designed to be effective against both alleles present – determined by 5′ RACE analysis for *Hoxd1* and comparison with available EST data for *Hoxa1* and *Hoxb1*. Sequence of morpholinos is as follows: *Hoxa1*, MO1-5′CTCATCCTCCTCGCATAGTCCATCT, MO2-5′CTCGCATAGTCCATCTATCACTAGG; *Hoxb1*, MO1-5′AGGAACTCATTCTGCTATTGTCCAT, MO2-5′ATCTGCCAGTGATGGAGGAGGGTCA; *Hoxd1*, MO1-5′AGGAACCTGCTGATCCCTCAATCTT, MO2-5′TAGGTAGGAATTCATCTCTAGGGAG. Morpholinos and mRNAs were diluted in Gurdon's buffer (15 mM Tris pH 7.5, 88 mM NaCl, 1 mM KCl) and injected at the four-cell stage into both left blastomeres with *GFP* mRNA co-injected as a lineage tracer. Before fixation the GFP was checked to ensure that the injections were on the correct side. Amounts injected ranged from 5 ng to 30 ng of PG1 morpholinos and 7.5 ng to 60 ng of control morpholino. Whenever a comparison was made between single, double or triple MO injections the total amount of MO was equalised by adding control morpholino. Several combinations of the 1st and 2nd MOs were analysed with the *Krox20* and *Engrailed-2* probes and gave the same result. In all experiments control morpholino (standard control, Gene-Tools Inc.) was also injected and the embryos included in subsequent analyses, but this never gave different results from the non-injected controls. CS2+GFP (25 pg) and CS2+xHoxd1 (100 pg) were linearised with *Not*I and transcribed with Sp6 polymerase.

Detection of gene expression by in situ hybridisation

The whole mount in situ hybridisation protocol used was described previously (Wacker et al., 2004b), as modified from a previous protocol (Harland, 1991). Antisense, digoxigenin-labelled probes were: *Hoxd1* (Sive and Cheng, 1991); *Hoxa1*, *Hoxb1*, *Hoxc6*, *Hoxb9* (Wacker et al., 2004a); *Krox20* (Bradley et al., 1993); *Engrailed-2* (Hemmati-Brivanlou et al., 1991); *Nrp1* (Richter et al., 1990); *Gbx2* (von Bubnoff et al., 1996); *Xslug* (Mayor et al., 1995); *Xsnail* (Mayor et al., 1993); *dll4* (Papalopulu and Kintner, 1993); *Otx2* (Pannese et al., 1995); *Hoxa2* (Pasqualetti et al., 2000); *Myod* (Hopwood et al., 1989); EST clones from the I.M.A.G.E. Consortium [LLNL] cDNA library (Lennon et al., 1996), *Hoxa3* (IMAGE4405749), or the NIBB library, *Hoxd3* (XL012i13); *Hoxd4* (XL094l20); *Hoxa5* (XL045g13).

Neural antibody analysis and cartilage staining

After bleaching (80% methanol, 6% H_2O_2, 15 mM NaOH), stage 46 embryos were washed (4×30 minutes PBS+0.2% Tween), blocked (30 minutes PBS+ 0.2% Tween, 3% BSA) and incubated overnight at 4°C with the neural antibody 2G9 (Jones and Woodland, 1989). The embryos were then washed and incubated overnight at 4°C with a secondary antibody conjugated to the Cy5 fluorophore. After washing, the embryos were dehydrated and fixed step-wise in methanol (25%, 50%, 75%, 100%). Before analysis embryos were cleared in Murrays and the hindbrain was visualised using scanning confocal microscopy (Leica TCS-NT). To visualize the cartilage, stage 49 embryos were fixed and stained with Alcian Blue (Pasqualetti et al., 2000).

Results

Hox PG1 genes are expressed in overlapping domains throughout development

To gain as complete a picture as possible of the expression of the PG1 genes, and to determine the possibilities for overlap of function, we performed an extensive whole mount in situ hybridization study of Hox PG1 gene expression (Fig. 1). This is the first comparative study of all three genes, although there is data on aspects of the individual genes (Godsave et al., 1994; Kolm and Sive, 1995; Wacker et al., 2004a). All three PG1 Hox genes begin to be expressed during gastrulation, where they are seen in a ring around the blastopore, with a gap on the dorsal side. When this expression is compared with the expression of the forebrain/midbrain marker, *Otx2*, it can be seen that whilst none of the Hox genes' anterior expression reaches the midbrain, *Hoxb1* appears to be expressed more anteriorly than *Hoxa1* and *Hoxd1*, almost fusing with the *Otx2* domain (Fig. 1A,I,Q). As elongation procedes, differences in the gene expression patterns begin to arise. As early as stage 14, a stripe of *Hoxb1* expression in the future hindbrain is observed and this becomes further defined by stage 18 (Fig. 1K-N). *Hoxa1*

and *Hoxd1* on the other hand do not exhibit this early stripe of expression, although they are both expressed in the hindbrain region (Fig. 1E,U). Expression of *Hoxd1* thereafter decreases and from stage 26 there only remains faint expression in the pharyngeal arches and the tailbud (Fig. 1W,X). The hindbrain stripe of *Hoxb1* expression persists until at least stage 30, accompanied by faint expression in the pharyngeal arches (Fig. 1O,P). *Hoxa1* remains expressed in a comparatively large domain along the axis, including the pharyngeal arches. In addition, as has been previously reported (McClintock et al., 2002), we observed midbrain expression of *Hoxa1* at stage 26 and 30 (Fig. 1G,H). Thus, the Hox PG1 genes are expressed in expansive and overlapping domains throughout early development, and all three of them are expressed in the hindbrain while it is being specified and patterned (Melton et al., 2004), and at a later stage in the pharyngeal arches.

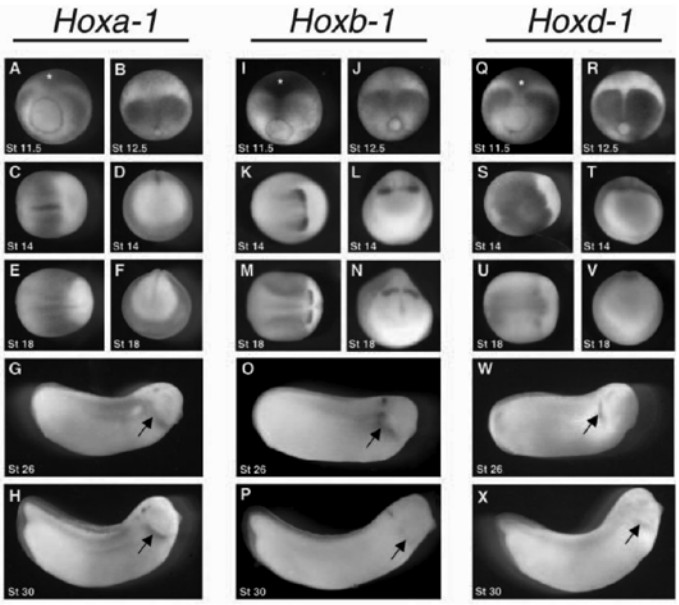

Fig. 1. Hox PG1 genes have overlapping expression domains during *Xenopus laevis* development. *Hoxa1* (A-H), *Hoxb1* (I-P) and *Hoxd1* (Q-X) have overlapping expression patterns. Gastrula stage embryos (A,B,I,J,Q,R) are shown from the vegetal side, dorsal up. When compared with Otx-2 expression (* in A,I,Q), it can be seen that the Hox expression does not reach the presumptive midbrain region. Neurula embryos are shown from the dorsal side, anterior to the right (C,E,K,M,S,U) or from the anterior side, dorsal up (D,F,L,N,T,V). Later embryos are shown from the lateral side, anterior to the right (G,H,O,P,W,X). Note pharyngeal arch expression of all three PG1 genes (arrows in G,H,O,P,W,X).

Knockdown of individual Hox PG1 genes leads to defects in rhombomere 4 formation

To analyse the role of the Hox PG1 genes we used the morpholino knockdown approach. Two morpholinos were designed for each PG1 gene and the effect on hindbrain patterning was investigated. To have an internal control, injections were performed at the four-cell stage into the left-hand side (lhs) of the embryo only (Fig. 2). For all three genes, both morpholinos gave the same phenotype, albeit at different concentrations (within a range of 10-30 ng), confirming the specificity of these morpholinos. The knockdown of each of the PG1 genes led to a defect in hindbrain patterning. For all three of them, r3 and r5 (shown by *Krox20* expression) were closer together on the injected side, indicating the reduction of r4, with the greatest reduction seen with the *Hoxa1* morpholinos (Fig. 2B,C). The expression domain of *Engrailed-2* however, a marker of the midbrain-hindbrain boundary (Hemmati-Brivanlou et al., 1991), was generally unaffected, although the width of this stripe often appeared to be smaller on the injected side.

For *Hoxa1* and *Hoxb1* this reflects the phenotypes observed in zebrafish and mice (Carpenter et al., 1993; Goddard et al., 1996; McClintock et al., 2002), but there is less information on the *Hoxd1* loss function phenotype. Therefore we decided to further check the specificity of the *Hoxd1* morpholino. To this end we tried to rescue the hindbrain phenotype with a *Xenopus Hoxd1* mRNA construct which lacks the 5′ UTR and therefore is not recognised by any of the morpholinos. Co-injection (in the lhs) of *Hoxd1* mRNA rescued the *Hoxd1* morpholino phenotype, as shown by Krox20 expression (Fig. 2K,L). Rhombomeres 3 and 4 were restored and in some cases were even larger than on the control side, as also seen in the simple *Hoxd1* overexpression phenotype (Fig. 2J). This demonstrates that the *Hoxd1* morpholino is specific and that in *Xenopus laevis*, *Hoxd1* has a role in hindbrain patterning.

Simultaneous knockdown of all three Hox PG-1 genes leads to severe hindbrain defects

Having established the specificity of the morpholinos we wanted to examine the effect of knocking down all three PG1 genes simultaneously. The morpholinos were injected (on the lhs) either in double combinations, or in the triple combination to remove all Hox PG1 function (Fig. 3). In all the double morpholino combinations the *Krox20* stripes are still present, although somewhat reduced and closer together (Fig. 3D,E,F). However, with the triple MO combination, *Krox20* expression in the hindbrain is either severely reduced or completely absent, with only a vestige of expression in the neural crest region (Fig. 3C). This indicates that an area encompassing at least r3-r5 is affected when all three PG1 genes are knocked down. The same effect was noted when the morpholinos were injected into the whole embryo (data not shown).

We attempted to rescue this phenotype with the morpholino insensitive *xHoxd1* expression construct (Fig. 4). When this

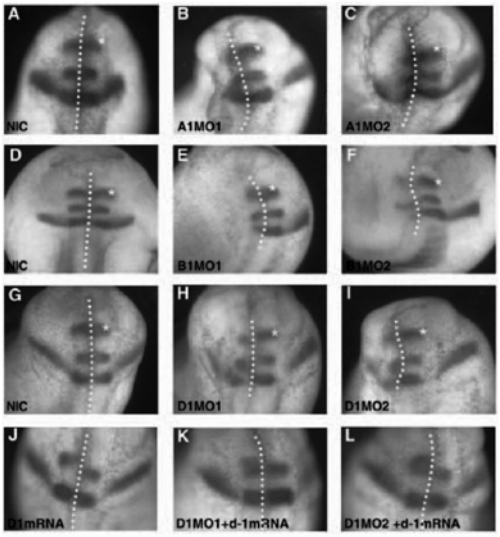

Fig. 2. Specificity of PG1 morpholinos. Two morpholinos for each Hox PG1 gene were injected into the left-hand side of the embryo and *Krox20* (r3 and r5) and *Engrailed-2* (midbrain/hindbrain boundary: * in A-I) expression analysed. The hindbrain region of early tailbud stage embryos (anterior to the top) is shown for non injected controls (NIC; A,D,G), *Hoxa1* 1st (B: 80%, *n*=15) and 2nd (C: 70%, *n*=10) morpholinos, *Hoxb1* 1st (E: 85%, *n*=13) and 2nd (F: 70%, *n*=20) morpholinos and *Hoxd1* 1st (H: 90%, *n*=11) and 2nd (I: 80%, *n*=15) morpholinos. Overexpression of *Hoxd1* morpholino insensitive RNA (J) and rescue of *Hoxd1* 1st and 2nd morpholinos with *Hoxd1* RNA is also shown (K,L). Dotted line indicates the midline.

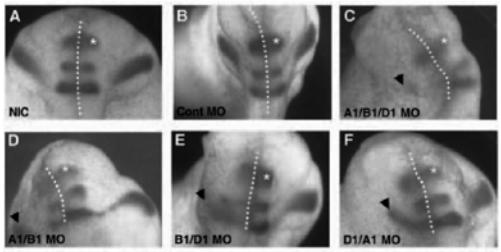

Fig. 3. The triple PG1 knockdown phenotype is more severe than the double knockdowns. Morpholinos for each Hox PG1 gene were injected either in double (D: A1/B1 52%, *n*=25; E: B1/D1 48%, *n*=29; F: D1/A1 64%, *n*=33) or triple (C: A1/B1/D1 94%, *n*=35) combinations into the left-hand side of the embryo and *Krox20* and *Engrailed-2* (*) expression analysed. The hindbrain region of early tailbud stage embryos is shown, with anterior to the top. Non-injected controls (NIC; A), and embryos injected on the left-hand side of the embryo with control morpholino (B) are also shown. Arrowheads indicate neural crest expression.

was co-injected with the triple MO combination the majority of embryos changed from having no *Krox20* expression, or only one faint narrow stripe, to having two stripes or one broad stripe spanning the r3-r5 region (Fig. 4E-G). This indicates that *xHoxd1* can partially rescue the triple knockdown phenotype and bring back some degree of patterning to the r3-r5 region of the hindbrain. We also attempted to rescue the phenotype with *Hoxa1* and *Hoxb1* constructs, but as the overexpression of these genes leads to a loss of the r3 *Krox20* stripe, interpretation of the rescue was difficult.

From these results, it appears that the phenotype of the triple PG1 MO embryos is not just an additive combination of the individual morpholino phenotypes, but is instead a synergistic effect. This indicates that there is a degree of redundancy between these genes in their hindbrain patterning function, and that any of the three genes can fulfil a basic hindbrain patterning role, even in the absence of the others. Therefore we concentrated on the triple PG1 MO combination for a more in depth analysis of Hox PG1 function.

Hox PG1 gene function is necessary for correct hindbrain patterning and segmentation

To further analyse the effect on hindbrain patterning of losing all PG1 function we carried out a detailed marker analysis of embryos injected with all three PG1 morpholinos (Fig. 5). It is clear that although hindbrain patterning is severely affected, as shown by the loss of *Krox20* stripes (Fig. 5G,H), the cells still adopt a neural fate, as the pan neural marker, *Nrp1* (Richter et al., 1990), is unaffected (Fig. 5A,B). In addition the most anterior domain of the embryo, expressing *Otx2* (Pannese et al., 1995) is not altered (Fig. 5C,D), and neither is the midbrain/hindbrain boundary, as shown by *Engrailed-2* expression (Fig. 3C). However, as we move posteriorly along the axis, a transformation of posterior hindbrain into anterior hindbrain becomes apparent. This is shown by the expansion of *Gbx2*. This gene has a stripe of expression at the boundary between the midbrain and hindbrain and in r1 (von Bubnoff et al., 1996), and on the side of the embryo injected with the PG1 morpholinos this stripe is expanded posteriorly (Fig. 5E,F). This posterior expansion was only partially rescued by co-injection of the *Hoxd1* mRNA (75% phenotype, reduced to 58%), indicating that at least one of the other Hox PG1 genes may be necessary for the restriction of *Gbx2* expression. More posterior hindbrain markers, such as *Krox20* and certain Hox genes (see following section), are either severely reduced or lost completely with the triple MO combination. Later analysis of the hindbrain using a neural specific antibody (Jones and Woodland, 1989) and confocal imaging indicated that not only was the patterning of the hindbrain affected, but also its morphology. In the triple MO injected side there were no clear rhombomere boundaries and the hindbrain appeared to be shorter and thinner compared with the control side (Fig. 5I,J). The midbrain also looked slightly affected but this could be a secondary effect due to the shortened hindbrain. Thus PG1 function is necessary for the correct segmentation of the hindbrain, as well as being involved in its patterning.

Another interesting aspect of the triple PG1 knockdown is the loss of segmented *MyoD* expression (Fig. 5H), indicating that the somites are also affected. This effect is specific and the mechanisms underlying it are currently under investigation.

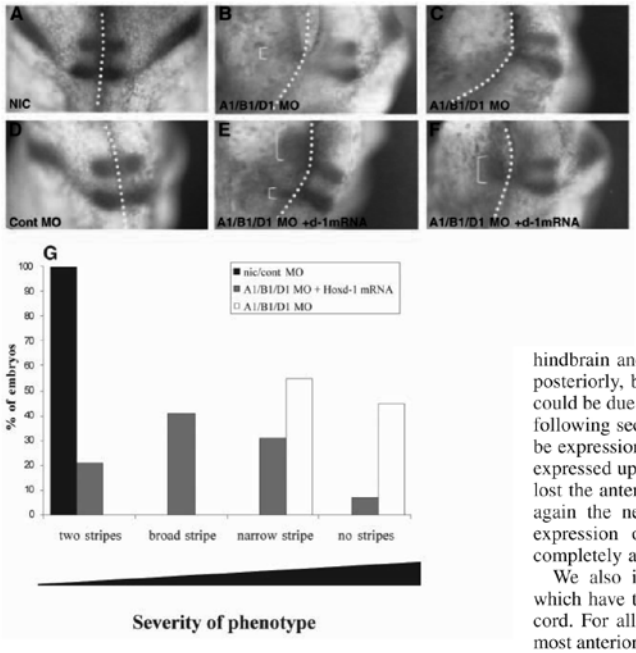

Fig. 4. *Hoxd1* mRNA can partially rescue the triple PG1 morpholino phenotype. Early tailbud stage embryos were injected on the left-hand side (lhs) with all three PG1 morpholinos (A1/B1/D1 MO) either alone (B,C), or in combination with *Hoxd1* mRNA (E,F). Non-injected controls (NIC) and control morpholino (Cont MO) are also shown (A,D). Anterior is to the top. G: *Krox-20* expression is partially rescued by *Hoxd1* RNA. Numbers of embryos with two stripes (E), one broad stripe (F), one narrow stripe (B) or no stripes (C) of *Krox20* expression were scored. Co-injection of *Hoxd1* RNA partially rescued the *Krox20* expression (n=29), which was either absent or very much reduced in embryos injected only with A1/B1/D1MO (n=18).

The function of Hox PG1 genes is necessary for the correct establishment of the 'Hox code'

To investigate whether the hindbrain defects that we observe could be due to a disruption in the 'Hox code', we analysed the expression of several Hox genes from different paralogous groups in embryos injected with the triple PG1 MO combination (Fig. 6). It is known that Hox-Hox cross-regulation occurs (Hooiveld et al., 1999), and as the PG1 genes are the first to be expressed in the embryo, and are expressed in a wide domain, they could play a role in the regulation of the other Hox genes. When we examined the expression of the PG1 genes themselves, *Hoxb1* and *Hoxa1* expression was downregulated (shown for *Hoxb1*, Fig. 6A,B), but the expression of *Hoxd1* was more variable, being reduced in only one third of embryos (n=24) and at other times being unaffected. This indicates that auto- and cross-regulation of these genes play a role in the maintenance of expression, but that external factors are also involved, particularly for *Hoxd1*.

When the other Hox genes were analysed it became apparent that the function of the PG1 genes is necessary for the establishment of the 'Hox code'. Both PG2 genes, *Hoxa2* and *Hoxb2*, normally expressed up to the anterior boundary of r2 and r3, respectively (Pasqualetti et al., 2000) were almost completely absent (shown for *Hoxa2*, Fig. 6C,D). For PG3 the effect was less uniform; *Hoxa3* and *b3* [expressed up to the r4/r5 boundary and in the neural crest derived cells of the 3rd pharyngeal arch (Godsave et al., 1994)] were severely reduced, with only the most posterior expression remaining (shown for *Hoxa3*, Fig. 6E,F). The effect on *Hoxd3* was less severe; its lateral stripe of expression was lost and its hindbrain and neural tube expression was fainter and shifted posteriorly, but slightly expanded laterally (Fig. 6G,H). This could be due to the inability of the neural crest to migrate (see following section) and thus the faint, expanded domain could be expression in the premigratory neural crest cells. *Hoxd4* is expressed up to the r6/r7 boundary and when PG1 function is lost the anterior expression domain is shifted posteriorly and again the neural crest stripe is lost (Fig. 6I,J). Thus, the expression of Hox genes in r2-r7 is either reduced or completely absent.

We also investigated several more posterior Hox genes, which have their anterior expression boundaries in the spinal cord. For all of these genes (*Hoxa5*, *Hoxc6* and *Hoxb9*) the most anterior, spinal cord expression was lost in the triple PG1 knockdown, but the diffuse mesodermal expression (in particular of *Hoxa5* and *Hoxc6*), and more posterior spinal cord expression, was unaffected (Fig. 6K-P).

To establish the specificity of this effect of the morpholinos, and to determine the degree to which reintroducing just one of the Hox PG1 genes would restore the Hox code, we utilised the *Hoxd1* morpholino-insensitive mRNA (Fig. 7). When this was injected alone, it induced anterior expansion of the expression of *Hoxb2* (Fig. 7A), but the other Hox genes analysed (*Hoxa3*, *Hoxd4* and *Hoxc6*) were unaffected. When *Hoxd1* mRNA was co-injected with the triple PG1 morpholino combination, however, it rescued the expression patterns of all of these Hox genes (Fig. 7C,F,I,L). This indicates that there may be a degree of redundancy between the PG1 genes and this is borne out by preliminary analyses of injections of the single morpholinos (Fig. 8). These showed that *Hoxa2* and *Hoxb2* expression was still present in all of the single knockdowns (shown for *Hoxb2*, Fig. 8A-D), although *Hoxb2* was slightly reduced with the *Hoxa1* and *Hoxd1* morpholinos. *Hoxa3* hindbrain expression however was reduced with the *Hoxa1* morpholino, and not affected by the other individual morpholinos despite being rescued in the triple PG1 knockdown by the *Hoxd1* mRNA (Fig. 8E-H, Fig. 7D-F). *Hoxd4* was affected by the *Hoxd1* morpholino, which appeared to give a similar phenotype to the triple combination (Fig. 8I-L). Thus different Hox genes appear to be dependant on different Hox PG1 genes, or a combination thereof, but these interactions will require further investigation before they are fully elucidated. However, these data do indicate that PG1 function in general is necessary for the establishment or maintenance of the expression patterns of both anterior and posterior Hox genes. For the more posterior genes this

requirement is restricted to the anterior, ectodermal expression domains, and the expression in the mesoderm and posterior ectoderm is unaffected.

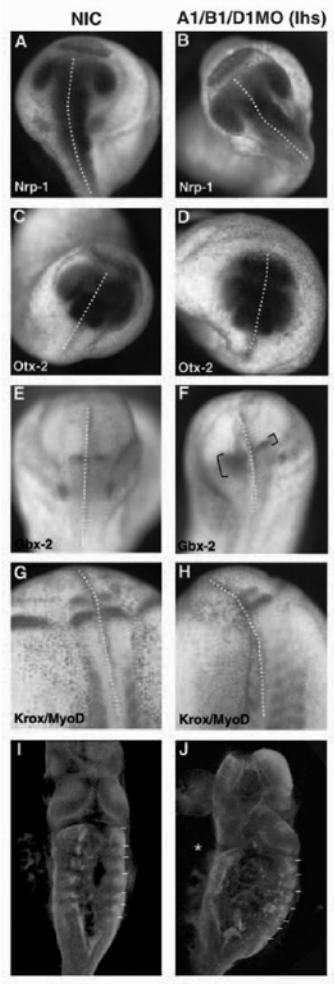

Fig. 5. Triple PG1 knockdown severely affects hindbrain patterning. Morpholinos for each of the Hox PG1 genes (A1/B1/D1MO) were co-injected into the lhs of the embryo (B,D,F,H,J). Non-injected controls are also shown (A,C,E,G,I). The hindbrain region is shown, with anterior to the top. Expression of the neural marker *Nrp1* (A,B: 100%, n=11), the anterior marker *Otx2* (C,D: 100%, n=10), *Gbx2*, which is expressed in r1 (E,F: 77%, n=13) and *Krox20* (G,H: no stripes 41%; one faint stripe 35%, n=71), was analysed. Dotted line indicates the midline. In later embryos (st. 46), the neural antibody 2-G9 was used to show the morphology of the hindbrain [NIC, I; A1/B1/D1MO, injected on the left-hand side (lhs) *, J: 100%, n=5]. Rhombomere boundaries are marked.

A role for PG1 genes in neural crest development

As Hox PG1 genes are expressed in the cranial neural crest, and PG1 function is clearly also necessary for the expression of certain other Hox genes in this region, we decided to investigate the effects of PG1 knockdown on neural crest and its derivatives. When we examined the expression of *Xslug*, which is expressed in all premigratory and migrating neural crest cells (Linker et al., 2000), we observed that on the triple MO injected side, the *Xslug* domain in the hindbrain region appeared to be upregulated compared with the control side (Fig. 9C). The expression, however, did not extend laterally but was restricted to an area close to the hindbrain. When the *Hoxd1* morpholino insensitive RNA was co-injected with the triple MO combination, the normal expression pattern was restored, with an additional expansion anteriorly (Fig. 9D). This is in keeping with the *Hoxd1* overexpression scenario, where the *Xslug* domain is expanded anteriorly (Fig. 9B). *Xsnail*, another neural crest marker, was also restricted to the area close to the neural tube but in this case the expression level was not significantly altered (Fig. 9E,F). These changes in expression could indicate a role for PG1 genes in neural crest cell migration, as in the knockdown situation the cells seem to remain close to the hindbrain, whereas when *Hoxd1* is overexpressed the *Xslug* positive cells are more widespread. It has been shown that *Xslug*, but not *Xsnail*, is downregulated when the neural crest cells reach their target (Linker et al., 2000). Therefore the apparent upregulation of *Xslug* compared with the control could be because the cells stay close to the hindbrain and thus *Xslug* expression is not downregulated. In later embryos expression of *dll4* in the pharyngeal arches (Papalopulu and Kintner, 1993) is severely reduced, whilst the expression of this gene in the forebrain and eye is still present (Fig. 9G,H). This indicates that PG1 gene function is vital for the development of the pharyngeal arches and this is confirmed by later analysis of the craniofacial structures using alcian blue staining. It can clearly be seen that, on the side injected with the triple PG1 MO combination, the gill cartilages, derived from the 3rd and 4th pharyngeal arches (the branchial arches) are completely missing (Fig. 9K). The ceratohyal, derived from the 2nd pharyngeal arch is still present, but reduced in size. Meckel's cartilage, derived from the 1st pharyngeal arch, is less affected but also appears to be slightly reduced. When the morpholino-insensitive *Hoxd1* RNA is co-injected, the gill cartilages are restored, but remain smaller than in the control side (Fig. 9L).

Thus, in the absence of Hox PG1 function, cranial neural crest cells are specified but they cannot migrate away from the hindbrain. Subsequent development of the pharyngeal arches and their derivatives is severely affected, with the complete loss of the gill cartilages.

Discussion

The complexity of the Hox gene family, and its members' redundancy and cross-reactivity, make it difficult to establish precisely what the function of each individual Hox gene is. However, there is increasing evidence that paralogous groups of Hox genes have shared functions. Therefore, rather than investigate individual Hox genes, we have taken a more global approach and studied the role of a complete paralogous group. The morpholino knockdown approach allowed us to block

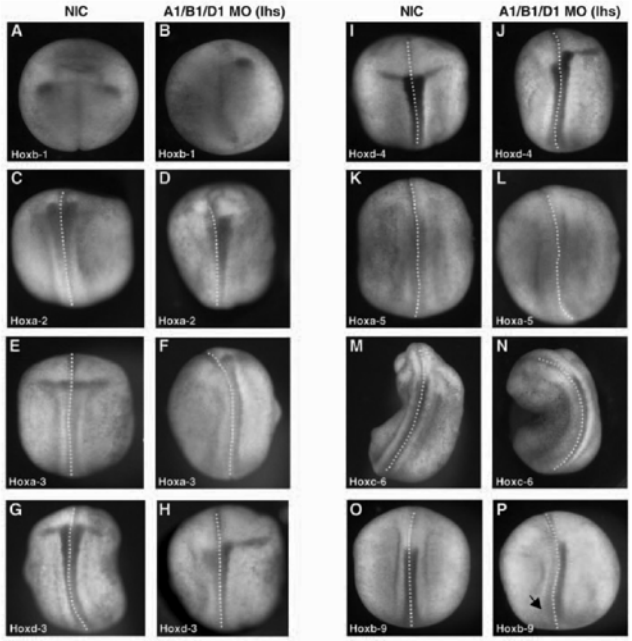

Fig. 6. Knockdown of all three PG1 genes affects more posterior Hox genes. Non injected controls (NIC; A,C,E,G,I,K,M,O) and embryos injected on the left-hand side (lhs) with all three Hox PG1 morpholinos (A1/B1/D1MO; B,D,F,H,J,L,N,P) were analysed for Hox gene expression at early tailbud stage. Embryos are shown from the dorsal side, anterior to the top. *Hoxb1* expression was lost (A,B: 70%, n=10), and *Hoxa2* (C,D: 100%, n=18) and *Hoxa3* (E,F: 100%, n=14) expression was almost completely absent. *Hoxd3* (G,H: 68%, n=19) and *Hoxd4* (I,J: 79%, n=14) neural tube expression was still present, but the pharyngeal arch expression was lost. The more posterior Hox genes (*Hoxa5*, K,L: 100%, n=10; *Hoxc6*, M,N: 71%, n=14; *Hoxb9*, O,P: 93%, n=14) had reduced neural tube expression in the anterior part of the embryo but their posterior expression domains (arrow) and mesodermal expression were unaffected.

simultaneously the translation of the three *Xenopus laevis* PG1 Hox genes, *Hoxa1*, *Hoxb1* and *Hoxd1*, and assess the roles of this paralogous group in the development of the embryo.

Hindbrain patterning and redundancy of Hox PG1 genes

The literature abounds with information about the effects on hindbrain patterning of single and double knockouts of Hox PG1 members. The data from mouse indicate that *Hoxa1* and *b1*, but not *Hoxd1*, are vital for the correct patterning and formation of the hindbrain and its derivatives. In this study we have shown that, in *Xenopus laevis*, all three Hox PG1 genes are expressed in the presumptive hindbrain region at a time when they could influence hindbrain patterning. This is in contradiction to the situation in mouse, where *Hoxd1* is only expressed in the mesoderm and neural crest (Frohman and Martin, 1992). However, the observed *Hoxd1* expression in the presumptive hindbrain region in *Xenopus* is very low, and is downregulated earlier than the other two paralogous genes, and it is therefore possible that a comparable low level expression in mouse was below detection limits.

When we knock down the function of all three PG1 genes we observe a more severe effect than in either the single or double knockdowns. Fig. 10 shows a schematic for the effect on the hindbrain of the stepwise elimination of Hox PG1 genes, with increasingly more severe phenotypes occurring when there is less PG1 function present. In *Xenopus*, the hindbrain expression of the r3/r5 marker, *Krox20*, is completely lost in the triple knockdown, whereas its expression domain is merely altered (indicating a reduction in the r3-r5 region) when at least one PG1 gene remains functional. Preliminary results indicated that the triple knockdown phenotype is a synergistic, rather than an additive effect, which implies a degree of redundancy between the PG1 genes and, thus, we concentrated on the triple knockdown to elucidate the basic PG1 group function. We cannot therefore rule out effects being due to the double morpholino combinations. However, defects in the hindbrain of *Xenopus* embryos completely deficient in PG1 function were more extensive than any previously reported for single or double PG1 knockouts in other organisms. Expression of markers for r2-r7 was downregulated in the triple knockdown, whereas the expression of an r1 marker, *Gbx-2* was expanded posteriorly, indicating a possible transformation of more posterior hindbrain to an r1 type identity. Later morphological analysis showed that the hindbrain is reduced in size and segmentation is perturbed. This phenotype is actually similar to the situation in zebrafish when the functions of Hox cofactors from the *Meis* and *Pbx* families are compromised (Fig. 10). In *Pbx4* (*lazarus*) mutants that have been injected with a *Pbx2* morpholino (to achieve a total block of early Pbx function) the hindbrain is not segmented and r2-r7 acquire an r1-like identity, referred to as the hindbrain ground state (Waskiewicz et al., 2002). Likewise, when the function of *Meis* is blocked, using two dominant-negative constructs, a similar, non-segmented hindbrain is produced (Choe and Sagerstrom, 2004). The similarity of these phenotypes to the triple PG1 MO phenotype suggests that they could, at least partially, be due to the blocking of PG1 gene function. A recent study in *Xenopus* showed that hindbrain *Krox20* stripes were eliminated when either a *Meis* morpholino or a dominant-negative *Hoxd1* RNA construct (which may also block *Hoxa1*

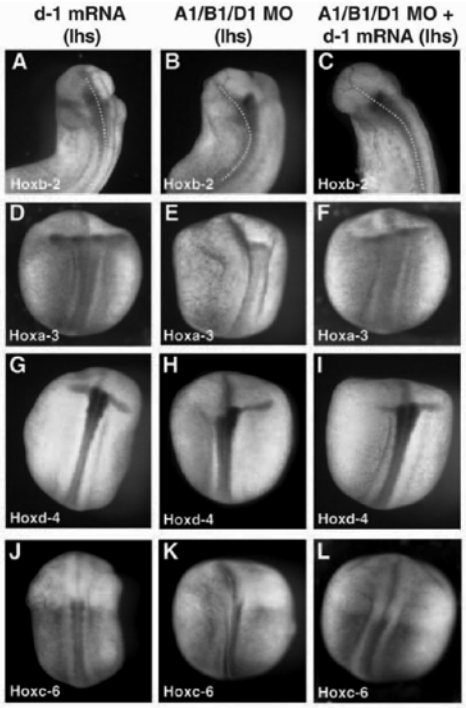

Fig. 7. *Hoxd1* mRNA rescues Hox expression in PG1 knockdown embryos. Embryos injected on the left-hand side (lhs) with *Hoxd1* MO insensitive mRNA (A,D,G,J), all three PG1 morpholinos (B,E,H,K) or a combination of both (C,F,I,L) were analysed for expression of *Hoxb2, Hoxa3, Hoxd4* and *Hoxc6*. Embryos are shown from the dorsal side, anterior to the top. *Hoxb2* expression was expanded posteriorly with *Hoxd1* mRNA (A: 75%, *n*=12), lost with the triple PG1 morpholinos (B: 93%, *n*=28) and rescued when a combination was injected (C: 48% of embryos had downregulated expression, *n*=33). The other Hox genes were not affected by the *Hoxd1* mRNA overexpression. PG1 MO downregulated expression of *Hoxa3* (E: 78%, *n*=9), *Hoxd4* (H: 100%, *n*=9) and *Hoxc6* (K: 78%, *n*=9) and in the rescues this downregulation was reduced to 34%, 20% and 33% for *Hoxa3*, *Hoxd4* and *Hoxc6*, respectively (F,I,L).

and *Hoxb1* function and, thus, be similar to the triple MO situation) were injected into the embryo (Dibner et al., 2004). It has also been noted in one *Hoxa1* knockout study in mouse that segmentation is completely blocked, but again all three of the PG1 genes could be affected, as the truncated *Hoxa1* splice variant still present could perhaps act as a dominant-negative (Chisaka et al., 1992).

The PG1 function in hindbrain could be mediated via other, downstream, Hox genes. For example, when both Hox PG2 genes are knocked out in mouse the rhombomere boundaries in the r2/r3 region are absent (Davenne et al., 1999). Thus the effect that we see with the triple PG1 knockdown on segmentation in this anterior region of the hindbrain (where PG1 genes are not expressed) could be due to the downregulation of *Hoxa2* and *Hoxb2*.

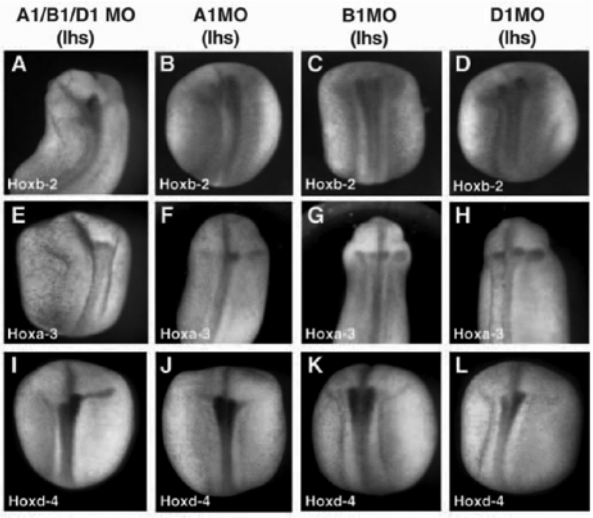

Fig. 8. Effects of single PG1 knockdowns on Hox genes. Embryos injected on the left-hand side (lhs) with the triple PG1 MO combination had reduced *Hoxb2* (A: 93%, *n*=28), *Hoxa3* (E: 100%, *n*=14) and *Hoxd4* (I: 79%, *n*=14). The *Hoxa1* MO weakly downregulated *Hoxb2* (B: 80%, *n*=10) and reduced *Hoxa3* (F: 54%, *n*=11), but not *Hoxd4* (J: 100%, *n*=20). The *Hoxb1* MO had no effect on any of these genes (C: *Hoxb2*, 100%, *n*=10; G: *Hoxa3*, 100% *n*=9; K: *Hoxd4*, 100%, *n*=18). *Hoxd1* MO reduced *Hoxd4* expression (L: 100%, *n*=16), weakly reduced *Hoxb2* (D: 43%, *n*=14), but did not affect *Hoxa3* (H: 100%, *n*=10).

PG1 Hox genes are necessary for the anterior portion of the 'Hox code'

Numerous interactions between various Hox genes have been shown to exist in the embryo (Deschamps et al., 1999; Hooiveld et al., 1999; Maconochie et al., 1997; Melton et al., 2004). Indeed one aspect of colinearity could be the sequential activation of anterior Hox genes that are, in turn, responsible for the activation of the more posterior ones (Hooiveld et al., 1999). Although the majority of the literature in mouse does not support this model, this could be due to a high degree of redundancy between different Hox genes masking the effect in single or double knockout mice. As the PG1 genes are the first to be expressed in the embryo we investigated the possibility that they could initiate a cascade of Hox expression, which would lead to the established 'Hox code'. Although PG1 function is clearly not a prerequisite *per se* for Hox gene

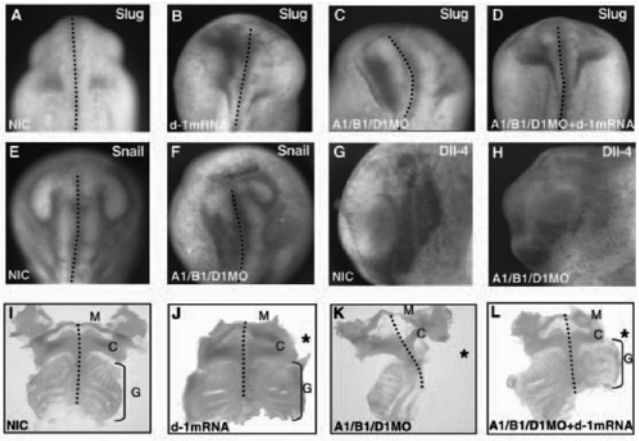

Fig. 9. Knockdown of all three PG1 genes affects the development of neural crest and its derivatives. Non-injected controls (NIC; A,E,G,I) and embryos injected on the left-hand side with *Hoxd1* MO insensitive mRNA (B,J), all three Hox PG1 morpholinos (A1/B1/D1MO; C,F,H,K) or a combination of both (D,L) were analyzed for neural crest gene expression, or stained with alcian blue to show the craniofacial structures. The embryos shown in A-F are early tailbud stage embryos shown from the dorsal side with anterior to the top. With the PG1 MOs, neural crest cells fail to migrate away from the hindbrain, as shown by *Xslug* (C: 86%, n=29) and *Xsnail* (F: 100%, n=14) expression. This is rescued (22% of embryos with restricted *Xslug* expression, n=9) by *Hoxd1* overexpression (D) and simple overexpression leads to an expanded *Xslug* domain (B: 100%, n=10). Slightly later tailbud embryos analyzed for *dll4* are shown from the left-hand side, with anterior to the left, and in the triple PG1 knockdown *dll4* expression in the branchial arches is restricted to close to the hindbrain (50%) or completely lost (G,H: 44%, n=16). Alcian blue was used to stain the cartilage of stage 49, embryos (I-L) shown from the ventral side to show the craniofacial structures (injected side on the right:*). The complete gill area is missing in the triple morpholino injected embryos (K: 82%, n=11) and this is rescued (26% of embryos with missing gill, n=19) when *Hoxd1* mRNA is co-injected (L). C, ceratohyal; G, gill cartilage; M, Meckel's cartilage. Dotted line indicates midline.

expression we did uncover a reliance on PG1 function for the correct expression of Hox genes from paralogous groups 2-9. This widespread effect on posterior Hox genes is more severe than that observed in the single or double PG1 knockouts in mouse (Dolle et al., 1993; Maconochie et al., 1997; Rossel and Capecchi, 1999). However, as the triple PG1 knockdown has never previously been investigated, it is possible that the situation in mouse is similar to that in *Xenopus*, with a high degree of functional redundancy between the genes.

From our data we cannot distinguish when the PG1 genes are required for the expression of the Hox genes examined, although it is likely to be an early input, as the later domains of *Hoxa2* and *b2*, at least, extend beyond the Hox PG1 expression domains. It is clear, however, that without Hox PG1 function the 'Hox code' is severely compromised. Thus, via the control of the 'Hox code', the PG1 genes can eventually exert their influence outside of their own expression domains. This has already been shown for *Hoxa1*, the knockout of which has defects in the r3 domain, which is anterior to the normal *Hoxa1* expression domain (Helmbacher et al., 1998). This mechanism would account for the extent of the defects that we observe in the triple PG1 knockdown. It could also be due to a more anterior expression domain early in development of one or all of the Hox genes, which could be the case for *Hoxb1*, as its early expression is very close to the *Otx-2* domain. Alternatively, these effects could be due to a non cell-autonomous effect. Several signalling pathways influence patterning of the hindbrain, such as the FGF and retinoic acid pathway (Roy and Sagerstrom, 2004; Walshe et al., 2002), and it is feasible that the extent of the phenotypes that we observe may be partly due to a disruption in these signalling pathways. It has also been shown in vitro that certain Hox proteins are able to cross the cell membrane (Amsellem et al., 2003; Chatelin et al., 1996) and thus it is possible that the Hox proteins themselves act non cell-autonomously to pattern the hindbrain.

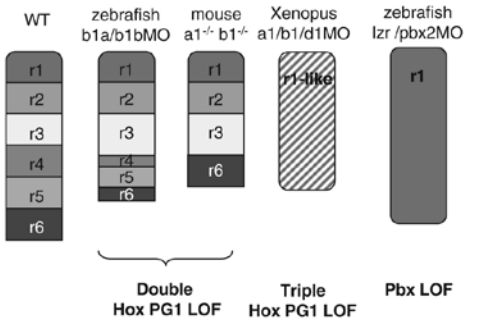

Fig. 10. Schematic for the patterning of rhombomeres 1-6 in the hindbrain in various loss of function (LOF) combinations of Hox PG1 genes, and also the Hox cofactor *Pbx*. There is a successive loss of rhombomeric identity as more PG1 function is removed in the different organisms: zebrafish (McClintock et al., 2002); mouse (Rossel and Capecchi, 1999); and *Xenopus* (this study). The triple PG1 LOF phenotype in *Xenopus* resembles the *Pbx* complete LOF in zebrafish (Waskiewicz et al., 2002).

A role for PG1 genes in neural crest migration and development of the pharyngeal arches

A link between the Hox PG1 genes and craniofacial development has previously been demonstrated, with *Hoxa1* and *-b1* double mutants lacking the 2nd pharyngeal arch and its derivatives as well as some derivatives of the 1st pharyngeal arch (Gavalas et al., 1998; Rossel and Capecchi, 1999). Here we show that in the absence of any PG1 gene function, cranial neural crest cells are still specified but they appear to be unable to migrate away from the neural tube. Conversely, when *Hoxd1*

is overexpressed, migration seems to be enhanced, as seen by the widespread *Xslug* domain.

It is interesting to note that in the *Hoxa1/b1* double knockout in mouse, neural crest cells fail to delaminate from the presumptive r4 territory, although they delaminate normally from the other rhombomeres (Gavalas et al., 2001). This failure could be more widespread in the triple PG1 knockdown, leading to a wholesale block of cranial neural crest cell migration. This could be due to the effect on the Hox code, as many of the Hox genes downregulated are also expressed in the neural crest, or are known to be involved in their patterning (Trainor and Krumlauf, 2001).

Major defects in the craniofacial structures are later seen in embryos lacking PG1 function, which are probably due to the inability of the cranial neural crest cells to migrate. Derivatives of all the pharyngeal arches are affected and most strikingly, the gill cartilages, derived from the 3rd and 4th pharyngeal arches, are completely missing. Effects such as this have not previously been seen in PG1 mutants, where generally only the 1st and 2nd pharyngeal arches are affected (Gavalas et al., 1998). Thus the complete abrogation of PG1 function has identified additional regions of the embryo which are ultimately dependant upon these genes for their formation.

In conclusion, we have knocked down the function of the complete Hox Paralogous Group 1 and illustrated that these genes have a degree of functional redundancy in their role of patterning the *Xenopus* hindbrain. We have demonstrated that PG1 function is essential for the correct establishment of the 'Hox code'. In the absence of PG1 function, and perhaps as a consequence of 'Hox code' disruption, hindbrain segmentation is perturbed and r2-r7 are mis-specified. In addition we have identified a novel requirement for Hox PG1 function in the migration of the cranial neural crest and the development of the pharyngeal arches.

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Chapter 5

HoxC6 is required for somitogenesis in *Xenopus*

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Abstract

Hox genes are involved in the regionalization of the vertebrate anteroposterior body axis In mouse, such a role has been shown for the *HoxC6* gene in the patterning of the axial skeleton. We investigated the developmental role of *Hoxc6* in *Xenopus laevis* and unexpectedly find a requirement for somitogenesis through regulation of the *Xenopus X-Delta-2*. This role contrasts sharply with the known functions of *Hox* genes in vertebrates and indicates a putatively ancestral, function for *Hoxc6* in *Xenopus*. Our observations challenge the current perception of the universality of *Hox* gene functioning in tetrapods and suggest that evolutionary changes in the amniote body plan have resulted in a different implementation of the *Hox* patterning system.

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Introduction

In vertebrates, the axial skeleton derives from metameric structures, called somites, appearing during body elongation on each side of the neural tube (Cinquin, 2007). These
somites are generated in an anterior to posterior sequence, at the anterior end of the unsegmented presomitic mesoderm (PSM) (Pourquié, 2002). Although somites are morphologically uniform, they will give rise to the different vertebrae specific for the axial level they are positioned.

Hox transcription factors are major players in conferring positional information along the anteroposterior axis in vertebrates (Krumlauf, 1994; Burke *et al.,* 1995; Lemons and McGinnis, 2006). The developmental roles of some *Hox* genes in vertebrates have been characterized by knockout and transgenic analysis in the mouse and by morpholino knockdowns in *Xenopus* and zebrafish. Single *Hox* knockouts do generally not lead to dramatic phenotypes (due to the high functional redundancy within the paralogous groups) but double, triple and quadruple knockouts have been very informative about the roles of *Hox* genes in development (McNulty *et al*., 2005; Wellik and Capecchi, 2003; McClintock *et al*., 2002; van den Akker *et al*., 2001; Horan *et al*., 1995a, 1995b). In the mesoderm, patterning by the *Hox* genes has been shown to underlie the regionalization of the axial skeleton and *Hox* genes are believed to specify the cervical, thoracic, lumbar, sacral and caudal anatomical regions (Kessel and Gruss, 1991). *Hox* phenotypes are in general interpreted as “homeotic transformations”, in which metameres adopt an identity normally associated with a different anteroposterior position. In the mouse, no *Hox* mutations are known to affect the basal underlying segmental organization of the trunk. However, a direct link between any *Hox* somitic domain of expression and any mutant phenotype has not yet been made. Nonetheless, it has been reported that *Hox* gene function is relevant within the presomitic mesoderm (McIntyre *et al.,* 2007). Thus, *Hox10* group function is required within the time window during which the thoracic/lumbar transition somites are being generated (Carapuço *et al*., 2005).

A link between body patterning and segmentation was first proposed in mouse. *Hoxd1* expression and *Hoxd3* expression were down-regulated in the somitomeres of the RBPjk-null mutant, suggesting that Notch signaling is somehow required for the correct expression of *Hox* genes during mouse somitogenesis (Zákány *et al.,* 2001). It has been shown that Notch mutants exhibit homeotic transformations and changes in vertebral identity in mouse and man (Cordes et *al.,* 2004; Turnpenny *et al*., 2007). In addition, in *Xenopus*, loss of function of the paralogous group 1 (PG1) leads to somitogenesis defects and a loss of

X-Delta-2 expression which already starts within the PSM before somite formation (Peres *et al*., 2006; Peres and Durston, 2006). Conversely, loss of function of *X-Delta-2* impairs early expression of *Hox* genes during gastrulation. This data shed light on a tight link between patterning and segmentation in *Xenopus*. Loss of PG1 function also leads to the downregulation of moreposterior *Hox* genes (McNulty *et al.,* 2005). Interestingly, none of the PG1 genes is expressed in the trunk somites at tadpole stages (except *Hoxa1* which is expressed in the most anterior somites) while more posterior *Hox* genes such as *Hoxb4* are expressed in those structures. It has been reported that *Hoxb4* ectopic expression affects somite formation in *Xenopus* without affecting other mesodermal derivatives (Harvey and Melton, 1988). Here, we investigate the potential link between the down-regulated *Hox* genes in the PG1 loss of function (as *Hoxc6*) and the somitogenesis process in *Xenopus*. We used a morpholino knockdown approach to disturb expression of several *Hox* genes in *Xenopus.* Our results show a requirement for *Hoxc6* function for a proper somitogenesis in *Xenopus*.

Results

Knockdown of *Hoxc6* leads to loss of segmentation

To investigate the role of *Hox* genes in a more primitive, anamniote tetrapod we performed loss of function by injecting morpholino antisense oligonucleotides into early blastomeres in *Xenopus laevis*. We injected morpholinos against *Hoxd1*, *Hoxb4* (unpublished data), *Hoxc6*, *Hoxb9*, *Hoxa7*, and *Hoxb7* (Peres personal communication). Injection with the anti *Hoxc6* morpholino, but not with any other morpholino against any one of the other *Hox* genes tested resulted in a clear and unexpected segmentation phenotype (Fig. 1 and 2). *Hoxc6* encodes two different proteins, a long form (LF) and a short form (SF) (Cho *et al.,* 1988). Our results show that the loss of the LF leads to a severe segmentation loss while the loss of the SF does not seem to affect somites formation (Fig. C and D). Segmentation of the frog PSM has been shown to be mediated by a Nocth ligand, *X-Delta-2* (Jen *et al*., 1997). *X-Delta-2* is expressed in the anterior half of a somitomere. Moreover, *X-Delta-2* was down-regulated upon PG1 genes knockdown in *Xenopus* (Peres *et al.,* 2006).

Thus, embryos were analyzed for expression for *X-Delta-2* and they showed loss of anterior somitic boundaries (Fig. 3). In addition, embryos were analyzed for *PAPC* (Kim et *al.,* 2000) and *Thylacine1* (Moreno and Kintner,2004) expression, both genes are expressed in the anterior part of the prospective somite (Fig. 4). These data confirmed the loss of anterior somite character. Analysis of a somite posterior marker, like *Uncx4.1* (Bussen *et al*., 2004), show a similar severe downregulation as seen for anterior markers as *X-Delta-2* (Fig. 3 and data not shown).

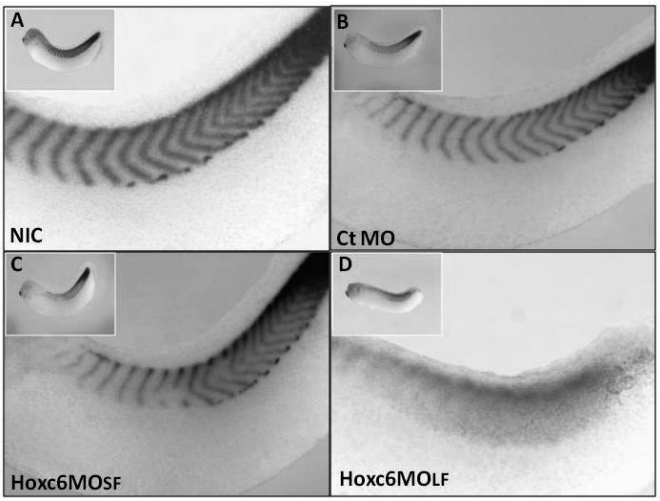

Figure1: *Hoxc6* knockdown leads to severe segmentation phenotype in *Xenopus*. Shown are uninjected control embryo (A), Control morpholino (CtMO) injected embryo (B), MO targeting the short form *Hoxc6* (C), and MO targeting the long form Hoxc6 (D) stained by whole mount in situ hybridization for the expression of *MyoD*. In D, note that the typical segmented pattern of *MyoD* is lost when the long form HoxC6 protein is lost.

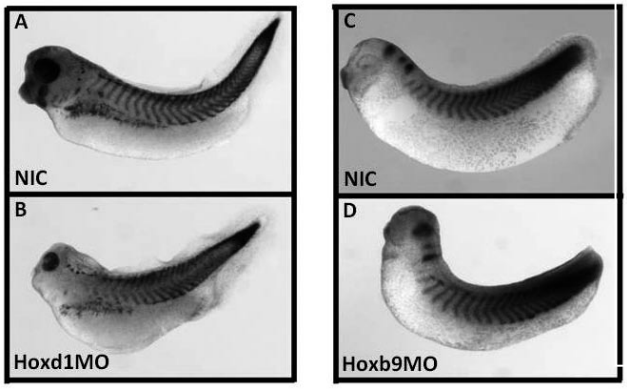

Figure 2: *Hoxd1* or *Hoxb9* knockdown does not affect trunk segmentation in *Xenopus*. Shown are uninjected control, *Hoxd1* MO injected, and *Hoxb9* MO injected stained by whole-mount in situ hybridization for *MyoD*, and *Engrailed2/Krox-20/MyoD*. Note that segmentation is unaffected in both cases.

Since it has been reported that *Mespo* (Joseph and Cassetta, 1999) is activated in the anterior PSM by *Tbx6* (Li *et al.*, 2006), we further analyzed *Hoxc6* LF morphans for the expression of these two markers. Their expression seems to be unchanged in the PSM (data not shown). Altogether, our results show that the loss of HOXC6 LF protein in *Xenopus* severely disturbs the polarity of the forming somites while the unsegmented PSM does not seem to be affected.

The loss of somites is specific for *HOXC6* long protein isoform

Our group previously reported the loss of segmentation upon the loss of PG1 *Hox* genes (Peres *et al*., 2006). This effect was through the loss of *X-Delta-2* expression during early development of *Xenopus*. Surprisingly, a single member of the PG1 genes, m*Hoxb1*, rescued *X-Delta-2* expression, but this was insufficient to restore a normal segmented phenotype. Here, we decided to investigate if other *Hox* genes (anterior and posterior to *Hoxc6*) were able to rescue the segmentation phenotype obtained by *Hoxc6* LF knockdown.

Figure 3: *X-Delta-2* expression is downregulated in the PSM after knocking down Hoxc6 long protein. *X-Delta-2* pattern in an uninjected control embryo (A) or control morpholino injected embryo (B) is dramatically lost after injection of *Hoxc6* MOLF. Note in C that the stripes are lost showing a loss of anterior half identity of the prospective

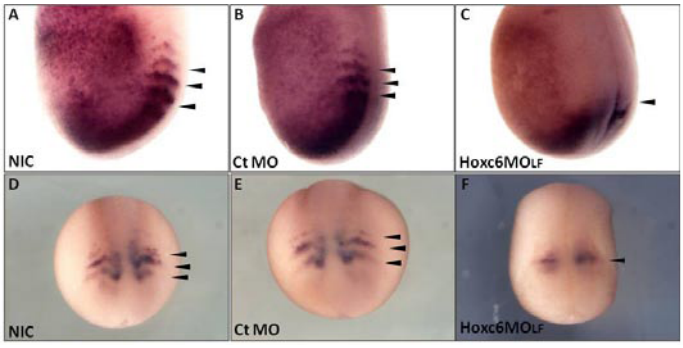

Figure 4: *PAPC* and *Thylacine 1* expression is downregulated in the PSM after knocking down Hoxc6 long protein. *PAPC* (A) and *Thylacine 1*(D) expression pattern in an uninjected control embryo or control morpholino injected embryo (B and E respectively) is dramatically lost after injection of *Hoxc6* MOLF (*PAPC* in C and *Thylacine1* in F). Note that in C and F the stripes are lost showing a loss of anterior half identity of the prospective somites after depletion of *Hoxc6*.

Surprisingly, members from the same paralogs 6, *Hoxa6* and *Hoxb6*, did not rescue the loss of segmentation (data not shown). The *Hoxc6* short form did not restore a proper

segmentation pattern either (Fig.5 D). Segmentation was not restored by neither *Hoxd1*, *Hoxa7* nor *Hoxb9* (Fig.5 C, E, F). However, *Hoxc6* coding for the long form protein rescued the segmented pattern. These results point towards a role of *Hoxc6* LF in segmentation.

The Drosophila homologue of *HoxC6* , *Antennapedia,* rescues the segmentation phenotype.

Hoxc6 was the first vertebrate gene cloned on the basis of its homology to the Drosophila gene, *Antennapedia* (*Antp*, (Carasco *et al.*, 1984)). We investigated whether Drosophila *Antp* could rescue the segmentation phenotype in *Xenopus* after depletion of Hoxc6 LF. Surprisingly, *Antp* mRNA seems to behave like *Hoxc6* LF mRNA and restores a segmented pattern (Fig. 6) while a segmentation gene, *Fushi taratzu* does not show the same behaviour (data not shown). These data suggest that a conserved function of *Hoxc6* mediates segmentation in the frog.

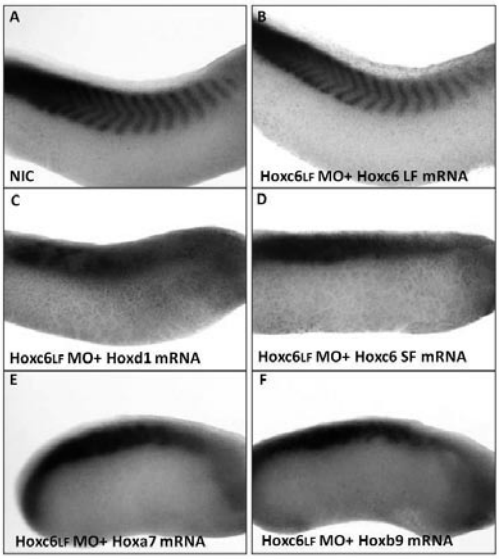

Figure 5: *Hoxc6* LF rescues segmentation defects while *Hoxd1*, *Hoxc6 SF*, *Hoxa7* and *Hoxb9* fail to rescue this phenotype. Is shown MyoD expression in an uninjected control tadpole (A), injected with MO targeting Hoxc6 LF +*Hoxd1* (C), +*Hoxc6* LF (B), +, *Hoxc6 SF*, + *Hoxa7* (E), + *Hoxb9* (F). Only *Hoxc6* mRNA encoding the long form Hoxc6 protein is able to rescue the segmentation loss.

HoxC6* targets *X-Delta-2

It has been previously shown that the PG1 *Hox* genes play a role during segmentation in *Xenopus* via *X-Delta-2* (Peres *et al*., 2006). PG1 genes knockdown reduces *X-Delta-2* expression at neurula stages. However, *X-Delta-2* expression was restored by

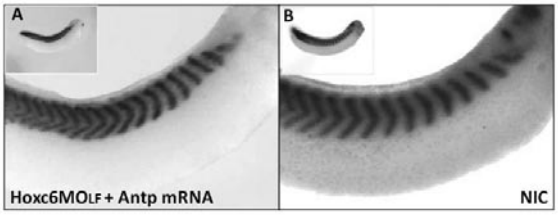

Figure 6: The *Drosophila* homolog of *Hoxc6*, *Antennapedia* rescues the segmentation phenotype induced by *Hoxc6* LF knockdown. MyoD expression is shown in uninjected control (B), and in MO+ *Antp* mRNA (A).

injection of the murine *Hoxb1*. This rescue was not sufficient to restore a segmented pattern at tadpole stages. In addition, we observed a severe downregulation of some posterior *Hox* genes such as *Hoxc6* upon PG1 genes loss of function. Here we investigated the potential link between *Hoxc6* and *X-Delta-2*. *Hoxc6* LF knockdown specifically downregulates *X-Delta-2* expression at early stages in *Xenopus* as mentioned above (Fig. 3) without affecting other mesodermal markers (data not shown). Conversely, ectopic expression of *Hoxc6* LF triggers upregulation of *X-Delta-2* expression within the PSM in agreement with microarray data (Michaut *et al*., manuscript in preparation). Moreover, *Hoxc6* LF mRNA as well as its Drosophila homologue, *Antp*, restore the typical pattern of *X-Delta-2* in the *Hoxc6* LF hypomorph (Fig. 7 and data not shown). These data suggest that *Hoxc6* could directly regulate the expression of *X-Delta-2* in the PSM in *Xenopus*, and that this function seems to be a conserved feature during evolution.

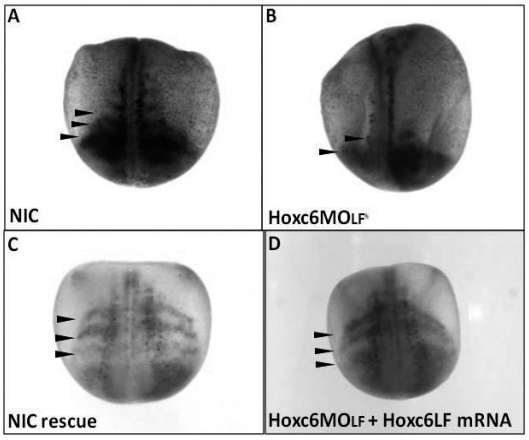

Figure 7: *Hoxc6* LF restores *X-Delta-2* expression pattern within the PSM. *X-Delta-2* expression with the stripes at neurula stage in an uninjected embryo (A, C), and in an embryo injected with Hoxc6MOLF (B), and in an embryo co-injected with MO targeting the LF *Hoxc6* and the mRNA insensitive to this morpholino (D). Upon *Hoxc6 LF* loss of function, *X-Delta-2* expression is downregulated as seen by the loss of the Delta stripes. The typical expression pattern is rescued by coinjection of Hoxc6 LF mRNA insensitive to the morpholino (D).

Discussion

In vertebrates, somitogenesis.results in the formation of metameric structures called somites. Despite their similarity, different somites will give rise to diverse vertebrae in the axial skeleton. *Hox* genes are among the major players in the specification of the morphological identity of the vertebrae (Krumlauf, 1994). The paraxial mesoderm starts to be patterned during gastrulation, prior to somite formation. The patterning process

continues later during somitogenesis. Moreover, a tight link between patterning and somitogenesis has been shown in *Xenopus* (Peres *et al.*, 2006) and mouse (Zákány *et al.*, 2001). In the frog, knockdown of the PG1 *Hox* genes leads to segmentation defects, as well as to downregulation of posterior *Hox* genes, like *Hoxc6*. The latter has been shown to be expressed within the somitic mesoderm (Oliver *et al.*, 1988). So, we investigated a potential link of *Hoxc6* and segmentation in *Xenopus*.

In this study, we show that *Hoxc6* long form (LF) function is required for *X-Delta-2* expression during *Xenopus* development. Our group has previously shown that loss of function of *X-Delta-2* in *Xenopus* leads to downregulation of *Hoxc6* at gastrula stages (Peres *et al*., 2006). This downregulation was rescued by *X-Delta-2* expression. These data and our results suggest a feedback loop between *X-Delta-2* and *Hoxc6*. In addition, these results show a requirement for Notch signaling for proper *Hox* expression in *Xenopus*. Indeed, *Hox* genes expression, *Hoxd1* and *Hoxd3*, was down-regulated in a Notch signaling mutant (Zákány *et al*., 2001). It was also suggested that the expression of *Hox* genes in the mouse PSM is crucial for specifying vertebral identity (Carapuço *et al.*, 2005), thus suggesting a tight regulation of *Hox* gene expression before somites formation.

Here in *Xenopus*, loss of function of *Hoxc6* LF severely disrupts segmentation, resulting in loss of somite identity. Indeed, anterior and posterior somite markers are lost resulting in the loss of boundaries between somites, and a proper segmentation pattern (Fig. 1). This phenotype is specific to *Hoxc6* LF because no other *Hox* genes (*Hoxd1*, *Hoxa7* and *Hoxb9*) could rescue the segmentation defects. Members from the paralogous 6 group did not rescue the segmentation loss. *Hoxc6* LF only could rescue the segmentation defects in *Xenopus.* These results suggest a difference in function between the two *Hoxc6* genes. It had already been shown that the two HOXC6 proteins show a differential expression pattern, strongly suggesting a difference in function (Oliver *et al*., 1988).

Whether *Hox* genes are upstream of somitogenesis and expression of *Delta-like* genes in higher vertebrates remains to be investigated. Moreover, the requirement of a single *Hox* gene for proper segmentation has not been reported up to date. Our study also showed that *X-Delta-2* is a putative direct target of *Hoxc6* LF. Indeed downregulation of *X-Delta-2* is restored upon *Hoxc6* injection in *Hoxc6LF* hypomorph. This function of *Hoxc6* is shared with its Drosophila homologue, *Antennapedia* suggesting an ancestral requirement of *Hoxc6* during segmentation in *Xenopus*. If *Xenopus* represents the ancestral condition of vertebrates, this could mean that there is either a complete dissociation of the pathway inducing or alternatively that the genetic pathway has been consolidated and that now multiple paralogous groups are directly upstream of *X-Delta-2*.

In PG1 loss of function, expression of posterior *Hox* genes has been shifted posteriorly or down-regulated. The latter is the case of *Hoxc6*. We speculate that loss of segmentation on PG1 could be through downregulation of *Hoxc6*, and subsequently *X-Delta-2*. *Hoxd1* and *mHoxb1* fail to rescue the segmentation defects in PG1 genes loss of function (Peres *et al.*, 2006). A similar phenotype is fully rescued by *Hoxc6* LF as we have shown. A rescue of PG1 segmentation phenotype by *Hoxc6* LF mRNA is likely to occur (currently under investigation). This would emphasize a predominant role of *Hoxc6* in *Xenopus* segmentation.

Experimental procedures

Embryos

Xenopus laevis embryos were stages according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). Culture of embryos and buffers were as described (McNulty *et al*., 2005).

Detection of gene expression by in situ hybridization

The whole mount in situ hybridization protocol used was a modified protocol from previously described (Harland, 1991). After in situ hybridization, embryos were bleached when necessary as previously reported (Song and Slack, 1994). Digoxigenin-labelled probes were made from the following plasmids: Krox-20 (Bradley *et al*., 1993); *Engrailed-2* (Hemmati-
Brivanlou *et al*., 1991); *MyoD* (Hopwood *et al*., 1989); X-Delta-2 (Jen *et al.*, 1997). *XTbx6*, *Mespo* and *Uncx4.1* were obtained from the NIBB Xenopus cDNA resource group.

Injection of morpholinos and mRNA

Morpholinos and mRNAs were diluted in Gurdon's buffer (15 mM Tris pH 7.5, 88 mM NaCl, 1 mM KCl) and injected at two cell stage. Morpholinos are as follows: Hoxd1 MO (McNulty *et al.*, 2005); Hoxc6LF MO: 5'-ATTCATATCTTCTCCTTTACCTGCC-3' ; Hoxc6 SF MO: 5'-TCTATTACAACACAAACCGGAGGTCG-3' ; Hoxb9 MO: 5'-TGACCGACCCACCAGCTTCTCCCCA -3' ; and the standard control morpholino (ctMO) from Genes-Tools Inc. (Gene-Tools Inc.). Hoxc6 MOs were used at concentrations

of 15 to 17,5ng per embryo; Hoxd1 MO as reported previously; Hoxb9 MO 20 ng; and ctMO up to 40ng.

The complete open reading frame of *Hoxc6* (BC084319) was amplified using primers including *BamHI* and *XhoI* restriction sites (F: 5'-**GGATCC**ATGAATTCCTATTTCACTAACCCTT-3'; R: 5'-**CTCGAG**GGGTGTCTCTCCATTCACTCTTT-3'). The short form *Hoxc6* was amplified using the following primers with *BamHI* and *EcoRI* restriction sites: (F: 5'-CG**GGATCC**ATGCTCACTAGCTGCAGGCAGA-3'; R: 5'-G**GAATTC**TCACTCTTTGCCTTGTCCCTCT-3'). After ligation of the PCR product into pGEM-T Easy vector (Promega), *Hoxc6* long form (LF, previously called PRII) was excised by *BamHI*/*XhoI* digestion and ligated into CS2+ vector (Turner and Weintraub 1994). The *Hoxc6* short form (SF, previously called PRI) was excised by EcoRI/BamHI and ligated to CS2+. These constructs were checked by sequencing. Since the LF construct does not encode the 3' sequences that are recognized by MO LF.

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Chapter 6

Vertical signaling involves Hox gene expression in the mesoderm

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Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

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

Results

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

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

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

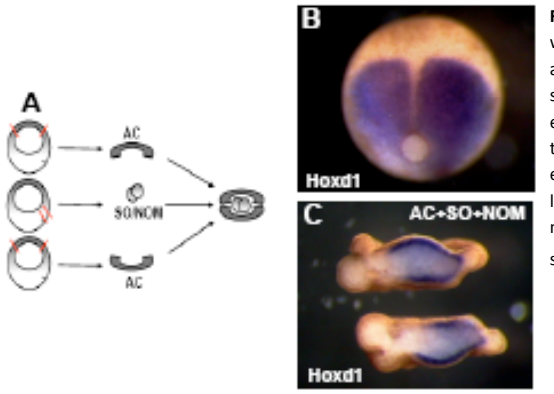


Figure 1: the wrap assay mimics signals within the embryo. **A**- a piece of NOM and a piece of SO are combined within a sandwich of 2 animal caps. **B**- *Hoxd1* expression in stage 12.5 embryo. Note that the SO area does not show expression. **C**- combination of NOM+SO leads to the expression of *Hoxd1*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

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

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

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

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

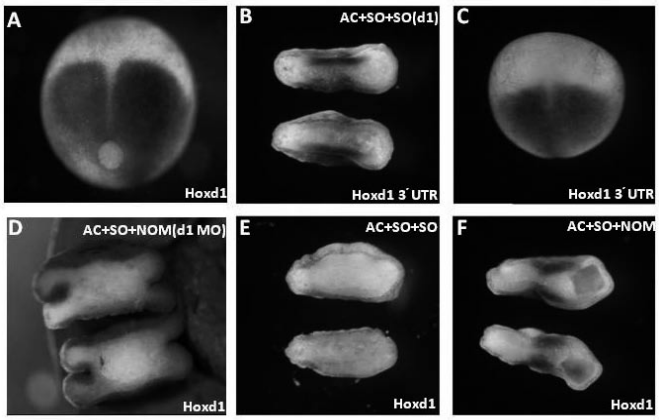


Figure2: *Hox* expression in the mesoderm is necessary and sufficient to its expression in the neurectoderm. **A**-*Hoxd1* expression at stage 12.5. **B**-wrap with SO+SO ectopically expressing *Hoxd1* show endogenous *Hoxd1* expression in the neurectoderm. **C**-*Hoxd1* 3´UTR expression. **D**-Knocking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

Vertical signalling and *Hox* gene transfer

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

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

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

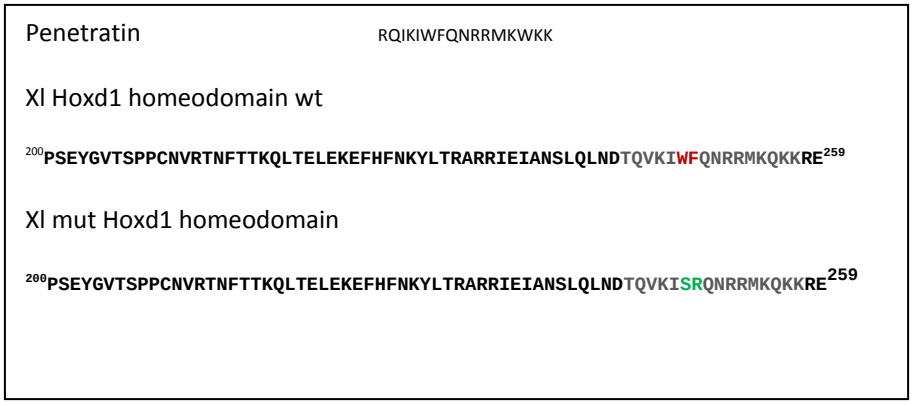


Figure3: Penetratin peptide and HOXd1 homeodomain. *Xenopus laevis* Hoxd1 exhibits a penetratin like peptide in its homeodomain. Amino-acids in red have been reported to be necessary for the transfer ability. In green are highlighted the mutant amino-acids in the penetratin like sequence.

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

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

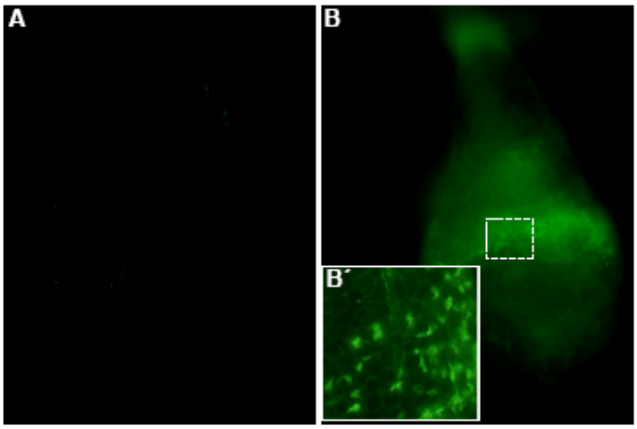


Figure 4: GFP linked to *Hoxd1* homeodomain efficiently enters cells of the wing imaginal disc. **A**-imaginal disc incubated with GFP. **B**-imaginal disc incubated with GFP liked to *Hoxd1* homeodomain (d1HD-wt-GFP). **B´**-close up of the plan in B. d1HD-wt-GFP enters efficiently the cells.

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

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

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

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

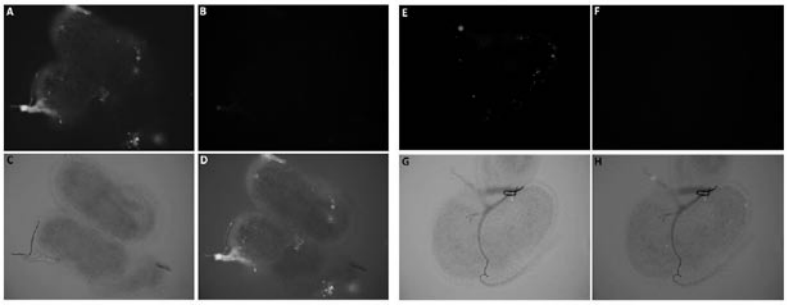
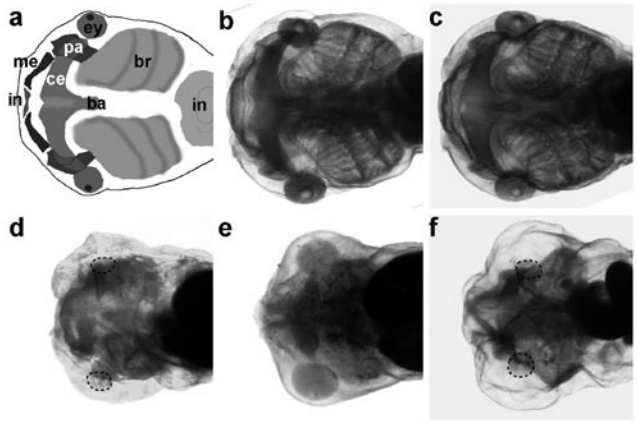


Figure 5: Mutation of two amino acids WF into SR within the penetratin like sequence abolishe the translocation from medium into cells. **A-D**, incubated imaginal discs with d1HD-GFP-wt in the medium. **A**,green chanel. **B**, red chanel. **C**, bright field. **D**, merge. **E-H**, incubated imaginal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

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

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

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



Discussion

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

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

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

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

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

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

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

Material and methods

Injection of morpholinos and mRNA

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

Detection of gene expression by *in situ* hybridization

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

Recombinant proteins synthesis

Preparation of His-tagged recombinant proteins were as described (Cao *et al*., 2004). XlHoxd1 OFR (McNulty *et al*., 2005) was amplified by the use of the following primers: **F**–CGGGATCCATGAATTCCTACCTAGAATA; and **R**-CCCAAGCTTCTAGGGTGAAGCGTCCTTGGA Primers for the synthesis of cargo protein Hoxd1 homeodomain (wild type or mutant) linked to the green fluorescent protein (GFP) are as follows:

F – GGGATCCATGCCCTGCAATGTGAGGACAAA; R – GGAATTCATTCCACCCCCTCCGCCACCCCTTTCCCTTTTCTTTTGTTTCA for the unmutated homeodomain (d1HD-GFP-wt); and for the mutated homeodomain, the primers are: F – GAAGATCTCTCGACAGAACAGAAGAATGAAACAAAA; R – CCCAAGCTTCTAGGGTGAAGCGTCCTTGGA

Imaginal discs preparation

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

Aknowledgments

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General Discussion and summary

The ordered emergence of structures in a developing embryo is an amazing intellectual challenge and inspiring food for the imagination; this phenomenon has fascinated investigators since the ancient Greeks. The publication, in 1924 by Spemann and his student Hilde Mangold of their discovery of the organizer and of its ability to instruct a secondary axis when grafted into a host embryo excited the imagination of developmental biologists. This initiated a "gold rush", which eventually (after more than 40 years) lead to the identification of molecules responsible for the organizer's properties. The surprise was that these were actually secreted antagonists for growth factors such as BMPs. In another breakthrough, attempts to understand patterning of the anterior-posterior (A-P) body axis lead to the discovery of a family of transcription factors: the *Hox* genes. The Drosophila *Antennapedia* complex was the first *Hox* locus discovered. Gene cloning in *Xenopus* revealed that certain sequences in *Hox* genes are extremely conserved throughout evolution. However, despite extensive investigations, *Hox* genes remain a very challenging topic in the field of developmental biology.

The aim of this thesis was to get more insight in understanding some of the Spemann organizer and *Hox* gene functions during *Xenopus laevis* development. We questioned the role of the Spemann organizer in patterning the main body axis (Chapter2). Because *Hox* genes are key players in body patterning, we investigated several aspects of their function. (Chapters3-5) First, we investigated the role of the first *Hox* genes expressed in the frog by loss of function of the complete *Hox1* paralogue group. We found a very interesting connection between the loss of function of these *Hox* genes and segmentation as well as expression of segmentation genes such as *X-Delta-2*. Interestingly, the loss of function of the first paralogue group leads to a defect in segmentation as well as to downregulation of certain more posterior *Hox* genes, including *Hoxc6*.

Thus, I decided to investigate the role of some of these more posterior genes during development of *Xenopus*. Loss of function (and conversely gain of function) of several posterior *Hox* genes showed surprisingly little effect on segmentation or the expression of segmentation genes. However, *Hoxc6* appeared to give a segmentation phenotype similar to the loss of function of the whole *Hox1* paralogue group (Chapter5). In addition, it appeared that ectopically expressing or down regulating *Hoxc6* also induced specific phenotypes during primary neurogenesis, indicating a function in this process (Chapter3).

In the frog, *Hox* gene expression starts in gastrula mesoderm in a temporally sequential manner. During the course of gastrulation, this temporal sequence appears to be

translated to generate a spatial pattern (Wacker *et al*., 2004a). A number of findings indicated that the mesodermal *Hox* gene expression sequence is somehow transferred to the gastrula's neurectoderm and that the time sequence of mesodermal movements in the gastrula causes mesoderm expressing early *Hox* genes to transfer their expression to a more anterior part of the neurectodermal axis, while mesoderm expressing progressively later *Hox* genes transfers expression to progressively more posterior parts of the neuraxis. In chapter 6, we have started to investigate this transfer of *Hox* gene expression from mesoderm to neurectoderm. We used gain and loss of function experiments in an *in vitro* system to confirm that the expression of the earliest expressed *Hox* gene, *Hoxd1*, in gastrula mesoderm actually is causal for the later expression of the same gene in neurectoderm. An unconventional Hox protein transfer was monitored for its possible relevance to the mechanism of this signaling process.

In chapter 2, we analyzed the role of the Spemann organizer in the A-P patterning of the trunk in *Xenopus laevis*. In the classical publications (Spemann and Mangold, 1924, 1936), the organizer was shown to be able to induce a secondary completely patterned axis when it is grafted into the ventral side of an amphibian embryo. Researchers have since then been able to show that the organizer secretes molecules antagonizing respectively one of following three classes of growth factors: Bone Morphogenetic Proteins (BMPs), Wnts and Nodals. This antagonism leads to the neuralization of the ectoderm and the appearance of dorsal neural plate. Besides secreting these antagonising signals, the organizer also affects development by self-differentiating and by regulating morphogenetic movements during gastrulation (reviewed in De Robertis *et al*., 2001). There is an evident function of the organizer in A-P patterning which has been proposed to reside in the existence of head, trunk and head organizers (Niehrs, 2004). On the other hand, neural induction, as a known major function of the organizer also connects to A-P patterning. It leads to the formation of anterior neural tissue (activation) which is progressively transformed into a more complete pattern including posterior neural tissue (transformation) (Nieuwkoop, 1952, De Robertis and Kuroda, 2004). Several signals have been shown to be implicated in this activation and transformation of neural tissue. Activation is believed to occur through signals from the organizer itself, while transforming signals appear to be derived from non-organizer mesoderm (Woo and Fraser, 1997; Kolm et al., 1997; Muhr et al., 1999; Wacker et al., 2004b). However the actual role of the organizer in establishing a proper A-P axis is pretty unclear. One of our ideas in Chapter 2 was to abolish all organizer functions by UV treatment of the *Xenopus* zygote (which ventralises the very early embryo and thereby blocks any development of the organizer) and then to restore them separately. We also investigated some of the different functions of the organizer separately by using an *in vitro* assay: the wrap assay (Jansen *et al*., 2007). This tissue recombination assay offers the possibility to manipulate different organizer signals and test their necessity for setting up an A-P pattern, in embryonic (neur)ectoderm.

It has been shown that if naive gastrula ectoderm (animal cap) is combined both with gastrula organizer mesoderm and with gastrula (*Hox* expressing) non-organiser mesoderm, this ectoderm expresses *Hox* genes and forms part of an A-P neurectodermal pattern. This system was used to analyse organizer function either by removing the organiser mesoderm and replacing it with molecular manipulations aimed at replacing specific organiser functions or by treating the system to block particular organiser functions. Neural activation by antagonizing BMP signaling, while the organiser and thus all of its other functions are absent, proved to permit the expression of A-P genes. Conversely, prevention of neural activation prevented the expression of several A-P genes tested. These results demonstrated that the role of the Spemann organizer is to induce competence in the neurectoderm to respond to transforming signals through its function of neural induction (activation) (**Chapter 2**). These data showed, for the first time, a clear function of the organizer in establishing the trunk A-P via an active process, neural activation. Our data also showed the capacity of a system such as the wrap assay as a paradigm for manipulating several sources of signals, and its usefulness for further investigations.

Axis formation, patterning and segmentation are intimately linked processes during development. It has been shown previously, in the mouse, that *Hox* gene expression is downregulated in a Notch effector mutant that is known to block segmentation (Zákány *et al*., 2001). In addition, it is also known that Notch mutants exhibit homeotic like transformations. These data suggest a tight link between segmentation and patterning. In *Xenopus*,PG1 *Hox* genes are the first to be expressed during development. These genes could thus play a major role in setting up a proper pattern in the main body axis and coordinating this patterning with processes like segmentation. In **Chapter 4,** we knocked down the whole paralogue 1 group (PG1). This lead to hindbrain segmentation defects as well as severe defects in trunk segmentation (Peres *et al*., 2006). It was previously demonstrated that *Hox* gene function in the hindbrain is linked to its segmentation (Guthrie, 2007; Lumsden *et al*., 2004; Dibner *et al*., 2004). Here for the first time, we reported a complete paralogue group knockdown and its connection to segmentation. In former work, a double PG1 knock out in mouse (Rossel and Capecchi, 1999) and knockdown of two PG1 genes in zebrafish (McClintock *et al*., 2002) had shown a progressive loss of rhombomeric identity. The triple PG1 knockdown in *Xenopus* showed a more severe hindbrain defect with the whole hindbrain being transformed to a rhombomere1 like identity with downregulation of several more posterior *Hox* genes belonging to PG's 2-6.

This loss of PG1 also induced a severe neural crest migration defects, presumably because members of PG1 need to be expressed in those migrating cells (Trainor and Krumlauf, 2001). More interestingly, loss of PG1 lead also to downregulation of *X-Delta-2*, and because of this, caused segmentation defects (Peres and Durston, 2006). Loss of function of

Delta-like genes had previously been shown to trigger segmentation defects in mice and men (Turnpenny *et al*., 2007; Kusumi et al., 1998; Dunwoodie et al., 1997). *X-Delta-2* is expressed from early during *Xenopus* development in an overlapping domain with *Hox* genes. It is interesting to see that the *Hox* genes down-regulated by PG1 loss are also expressed in the same general domain as *X-Delta-2*. I decided then to investigate the roles of those posterior *Hox* genes in segmentation.

In **Chapter 5,** knocking down several posterior *Hox* genes didn't seem to trigger any particular segmentation defects even though genes like *Hoxb4* are expressed in somites and it has been reported that *Hoxb4* ectopic expression affects somitogenesis in *Xenopus* (Harvey and Melton, 1988). However, *Hoxc6* loss of function triggered a severe segmentation defect. Our results show a clear connection between *X-Delta-2* and *Hoxc6* similarly as previously reported between *X-Delta-2* and PG1 (Peres and Durston, 2006). Loss of function of *Hoxc6* already leads to downregulation of *X-Delta-2* at gastrula stages, and the opposite is also true. We show that *Hoxc6* is required for proper trunk segmentation in *Xenopus*. This is the second time that *Hox* genes and segmentation have been shown to be connected during *Xenopus* development. I am currently trying to see if the PG1 loss of function segmentation phenotype can be rescued by *Hoxc6*. The expectation is that the segmentation phenotype in PG1genes loss of function is due to the severe downregulation of *Hoxc6*. A direct connection between a *Hox* mutant and segmentation has never so far been reported in the mouse.

However, it has been shown that the function of *Hox* genes is important within the presomitic mesoderm prior to somite formation (Carapuço *et al.,* 2005). The situation in *Xenopus* is similar: *Hox* genes are already expressed in mesoderm early in development (gastrulation), before somite formation (in the neurula at stage 17). Interestingly, *X-Delta-2*, a segmentation gene, is also expressed early in development at the same time as the first *Hox* genes (Peres *et al.,* 2006). Interaction between the somitogenesis clock and the "patterning clock" is suspected to occur prior to physical somite formation (Peres *et al*., 2006). The methodology for *Hoxc6* loss of function by knockdown can explain the severity of the phenotype obtained in the frog in comparison with that resulting from the classical mouse *Hoxc6* mutant. Indeed, a morpholino approach does not disturb any regulation at the DNA level while physical disruption of the DNA by a cassette insertion leads to different regulation of neighbouring genes and hence is likely to trigger a phenotype not reflecting a clear knock out mutant (Montavon *et al*., 2008). Moreover, the Drosophila homolog of *Hoxc6*, *Antennapedia*, is able to rescue the loss of segmentation that follows *Hoxc6* loss of function. This probably shows a more primitive function of a vertebrate *Antennapedia* orthologue. It would be interesting to see if Drosophila *labial* can rescue the phenotype of PG1 genes loss of function. In *Xenopus*, the first *Hox* gene expressed, *Hoxd1*, did rescue the downregulation of *X-Delta-2* upon PG1 loss of function but without restoring a

segmented pattern. This could reinforce the idea that segmentation defects in PG1 loss of function are somehow related to the downregulation of *Hoxc6*. Results from an attempt to rescue PG1 loss of function by *Hoxc6* could shed light on another function of a *Hox* gene beside its traditional patterning function.

Yet, another feature of *Hoxc6* morphans is a lack of response to the touch stimulus. Thus, I decided to monitor the expression of neural and neuronal markers in *Hoxc6* morphans. Our data show a severe loss of primary neurons when *Hoxc6* function is impaired. *X-Delta-2* is also a neuronal gene expressed in neurogenic regions from early stages of development (Peres and Durston, 2006). Extensive studies from Jessell's lab have provided strong evidence for a major role of HOX-C proteins in establishing the columnar structures within the spinal cord (reviewed in Di Sanguito *et al*., 2008). It looks as if members of the HoxC cluster like *Hoxc6* play a very important role in neurogenesis in the frog. In addition, ectopic expression of *Hoxc6* triggers the ectopic expression of *N-tubulin* (Oschwald *et al*., 1991). This result is very surprising, and shows for the first time a link between *Hoxc6* and formation of the primary neurons. I realized that *Hoxc6* and *N-tubulin* are already expressed in an overlapping domain during gastrulation. Indeed, previous work had shown that some genes belonging to the *N-tubulin* synexpression group were already expressed at very early stages (XPak3 another marker for primary neuronal differentiation is already expressed as mRNA in the egg for example, and XSeb-4 at early gastrula, Souopgui, thesis, 2002). Loss- and gain of function results place *Hoxc6* upstream of *N-tubulin* in the proneural cascade, although epistatic analysis with genes from the proneural pathway will answer this question definitively. It would very interesting to check a possible role of other *Hox* genes in neurogenesis in *Xenopus*. Indeed, a future line of research would be to knock down more *HoxC* genes and try to analyse neuron formation. It will be interesting to see if other *Hox* genes from other clusters will be able to affect the phenotypes of the neurons that are generated.

Hox genes are first expressed in marginal zone mesoderm in the *Xenopus* gastrula (Wacker *et al*., 2004a). During gastrulation, the mesoderm cells involutte and move under the gastrula ectoderm. Then, somehow the mesodermal *Hox* pattern is transferred to the overlying ectoderm: the presumptive neurectoderm shortly after these two tissues have been in contact (Wacker *et al*., 2004a). It has been shown that there are signals from the underlying mesoderm (called 'vertical signals') that act on the neurectoderm to pattern this tissue layer (Proznanski and Keller, 1997; Wacker *et al.*, 2004a). However, the exact nature of such signals is not well understood. It is noteworthy though that twenty years ago, Prochiantz'lab had demonstrated that the Antennapedia protein is able to cross cell membranes of several cell types under conditions excluding any endocytosis (Joliot *et al*., 1991). This peculiar behaviour seems to be a common feature of homeoproteins because of the existence of a signal peptide within the third helix of the homeodomain, the

penetratin peptide (see **chapter 6;** Joliot and Prochiantz, 2004). It was very tempting to imagine that this unconventional protein transfer could be happening in vivo and could be the basis of the vertical signalling responsible for posteriorizing the neurectoderm. It has been shown that hosted proteins are able to retain their activity, and can regulate targets in the host cell (Brunet *et al.,* 2005; reviewed in Prochiantz, 2003). We started the analysis of this question using the wrap assay because of the possibility to manipulate different components in this tissue recombination assay and because its mimics the embryonic situation. We demonstrated for the *Hox* gene *Hoxd1* that the expression of this *Hox* gene within the mesoderm is necessary and sufficient to induce its expression in the neurectoderm. These results pushed us to see whether the protein itself is involved in this process. We took advantage of recombinant protein technology to see whether some Hox proteins could indeed travel from cell to cell. We started the analysis with the first *Hox* gene expressed in *Xenopus*, *Hoxd1*. As previously reported for HOXB4, HOXD1 is able to cross an intact epithelium in Drosophila imaginal discs. Moreover, mutations of two amino-acids within the penetratin sequence abolished its capacity to translocate in accordance with previous in vitro data (Brunet *et al*., 2005). In addition, the full HOXD1 protein is able to translocate into *Xenopus* cells when injected into the blastocoel and then triggers a phenotype similar to that caused by its mRNA injection. This showed that our recombinant protein seems to retain its activity. The main difficulty with this project has been the limited knowledge of **direct** HOX targets (required to demonstrate activity of translocated Hox proteins) (Svingen and Tonissen, 2006). Our data about *Hoxc6* loss- and gain of function suggested that *X-Delta-2* is a very good candidate for being a possible direct target of *Hoxc6*. Thus, I am currently checking *X-Delta-2* expression after HOXC6 injection into the embryo, as well as after injection of an antibody recognizing the endogenous protein, and thus blocking the transfer. We have also incubated embryos with the recombinant HOXC6 protein and checked *X-Delta-2* and *N-tubulin* expression. Immunohistolocalization on slides is currently being used to follow the possible transfer of a tagged protein from mesoderm to ectoderm in the wrap system and in the whole embryo. I am really looking forward to the results of these investigations to see if Hox proteins could indeed play a "messenger protein" function as predicted by Prochiantz.

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Nederlandse Samenvatting

Hox genen vormen een belangrijke familie van transcriptie factoren betrokken bij de embryonale ontwikkeling van zowel vertebraten als invertebraten. Deze genfamilie bestaat in land vertebraten uit 39 Hox genen die in het genoom georganizeerd zijn in 4 clusters. De belangrijkste functie van deze genen is de regionalizatie van de kop-staart as (anterio-posterior) gedurende embryonale ontwikkeling. De correcte regulatie van deze genen zorgt ervoor dat structuren zoals bijvoorbeeld ribben en bepaalde neuronale connecties zich op de juiste plek van het lichaam ontwikkelen.
Ondanks dat deze genen al jaren onderwerp van intensief onderzoek zijn,worden nog met grote regelmaat belangrijke ontdekkingen gedaan die een beter inzicht geven in de ontwikkelingbiologische en evolutionaire achtergrond van de opbouw van het vertebrate “body plan”.
In dit proefscrhift wordt de rol van deze genfamilie nader onderzocht in de vroege ontwikkeling van de afrikaanse klauwkikker (*Xenopus laevis*). Deze soort ontwikkelt zich in veel aspecten representatief voor hogere gewervelden (dus muis, mens etc.) maar biedt praktische voordelen voor onderzoek. Verder is deze soort interessant omdat veel ontwikkelingspatronen primitiever zijn en dus inzicht kunnen bieden in primitieve en “derived” (nieuw ontwikkelde) aspecten van de het *Hox* gebaseerde patroonvormingssysteem.
In dit proefschift worden verschillende aspecten van het mechanisme van patroonvorming van *Hox* genen onderzocht. De rol van verschillende genen (*Hox-1* genen en *HoxC6*) wordt onderzocht door middel van morpholino gebaseerde uitschakeling en de consequenties van het verlies van deze

genen voor de otwikkeling van het embryo worden gekarakteriseerd. Hieruit blijkt onder andere dat de rol van *Hox-1* genen in de patroonvorming van de achterhersenen veel groter is dan verwacht op basis van knockouts in de muis.
Het onderzoek naar de rol van *HoxC6* laat zien dat dit gen onverwachts betrokken is bij de formatie van primary neurons en bij het proces van segmentatie (somitogenesis) van de *Xenopus* romp.
Het mechanisme dat er voor zorgt dat het typische patroon van *Hox* gen expressie langs de kop-staart as tot stand komt wordt onderzocht door middel van explantatie en transplantatie van embryonaal weefsel (een techniek die vrijwel alleen toegepast kan worden in *Xenopus*). Hieruit wordt een model herleid waarin het belangrijkste vroege embryonale signaal center (de Spemann organizer) samen met een *Hox* klok in het nog niet geïnvolueerde mesoderm verantwoordelijk is voor de vorming van de afzonderljike *Hox* expressie zones langs de anterio-posteriore as.
Ook blijkt het dat er een directe overdracht plaats vindt van *Hox* expressie in het mesoderm naar het neurectoderm, mogelijkerwijs door unconventionele *Hox* gen overdracht waarin het *Hox* protein zelf wordt overgedragen tussen cellen.
Deze thesis verschaft enkele nieuwe en onverwachtse inzichten in de rol van *Hox* genen in de vroege embryonale ontwikkeling. De belangrijkste bijdrage wordt waarschijnlijk geleverd door de vaststelling dat de functie van *Hox* genen, zoals *Hox-1* genen en *HoxC6*, veel breder is dan verwacht op basis van onderzoek gedaan in de muis.

List of publications (d.d. 11 November 2008)

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- **Bardine N***., Woltering J.M.*, Donow C., Schuff M.F., Knöchel W. and Durston A.J.

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- Schuff M*., **Bardine N***., Siegel D., Donow C. and Knöchel W.

Characterization of FoxO genes in Xenopus laevis. Manuscript in preparation.

- Woltering J.M., Vonk F.J., Müller H., **Bardine N**., Tuduce I.L., de Bakker M., Knöchel W.P., Sirbu I.O., Durston A.J. and Richardson M.K

Axial patterning in snakes and caecilians: evidence for an alternative interpretation of the Hox code . PNAS in revision.

Curriculum Vitae

Nabila Bardine was born on 3 January 1977 in Kasba Tadla (Morocco). She moved with her family to Marseilles, France, at the age of 10 where she attended secondary education and studied biology at the University of Luminy. She received her Master degree in 2002 for work done in Pierre-Marie Martin's lab on the role of adrenomedullin in tumor growth. In September 2002 she joined the group of Antony Durston at the Hubrecht laboratory, KNAW, to study *Hox* genes. She moved with this group to Leiden University in 2005 and briefly continued her research there. From 2006 to 2008 she has worked in the group of Walter Knöchel at Ulm University to finish the work for her thesis. The main work for this thesis has been to analyse the role of *Hoxc6* in *Xenopus laevis* embryogenesis. After the completion of her PhD she plans to continue in research.