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

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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 $\sim 30^\circ$ 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 antero-posterior (A-P) axes.

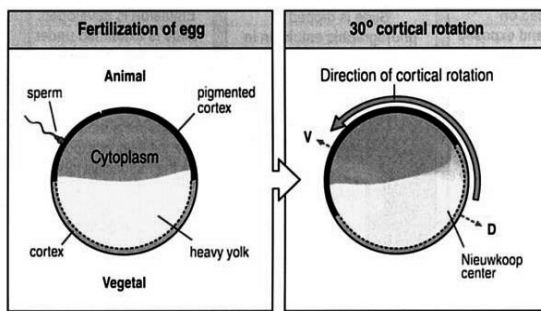


Figure 1: *Xenopus* egg fertilization leads to cortical rotation. The sperm entry at the animal pigmented pole triggers a loosening of the yolk from the cortex (left panel) which is followed by a cortical rotation. This results in displacement of maternal components from the vegetal pole towards the 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)

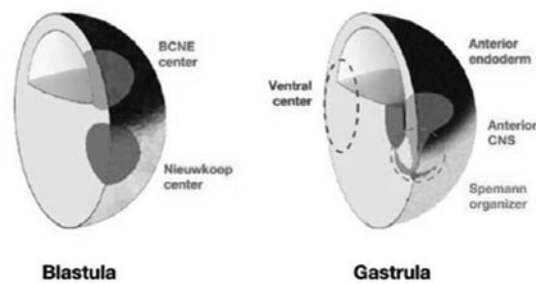


Figure 2: Important early signaling centers within the early embryo. Formation of the Nieuwkoop signaling center triggers the formation of the Blastula Chordin and Noggin Expressing region (BCNE) during the blastula stage. Mesoderm induction is accompanied by formation of the Spemann organizer dorsally. 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, misexpression 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 naïve 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 neur ectoderm 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).

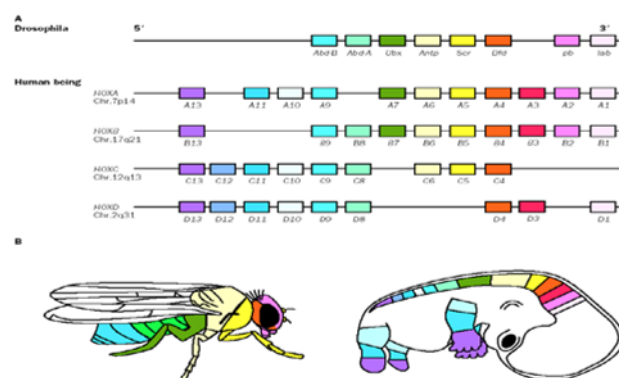


Figure 3: Chromosomal organisation of Hox clusters and their expression pattern in *Drosophila* and a human embryo. The fruit fly possesses a unique cluster while humans have four. Hox clusters are temporally and spatially colinear: genes located at the 3' end of the cluster and more rostrally expressed 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 (Durstion *et al.*, 1989; Sive *et al.*, 1999). This effect affects both the mesoderm and neurectoderm layers (Durstion *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 aforementioned 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 chromatin 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 clusters 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 Matisse 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; Gaubo *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 sympathetic 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 peripheral 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 presegmental mesoderm, PSM), and neur ectoderm (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 precursor) 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 Vasilias, 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

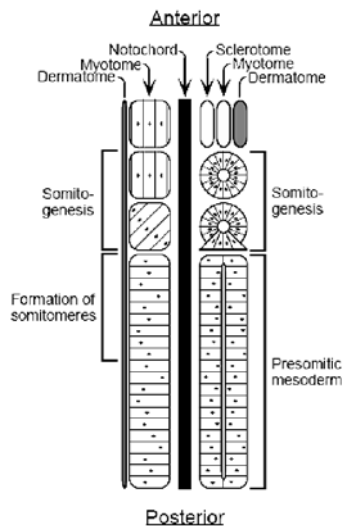


Figure 4: Modes of somites formation and their derivatives. In mouse and chick, somites form by budding off from the PSM and making epithelial spheres (right on the picture). Epithelial somites will differentiate into skin derivatives (dermatome), muscle derivatives (myotome) and bone derivatives (sclerotome). *Xenopus* shows a peculiar mode of segmentation. Anterior PSM cells detach from the posterior PSM by fission. In addition, they undergo a 90° rotation to end up in an 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*

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).

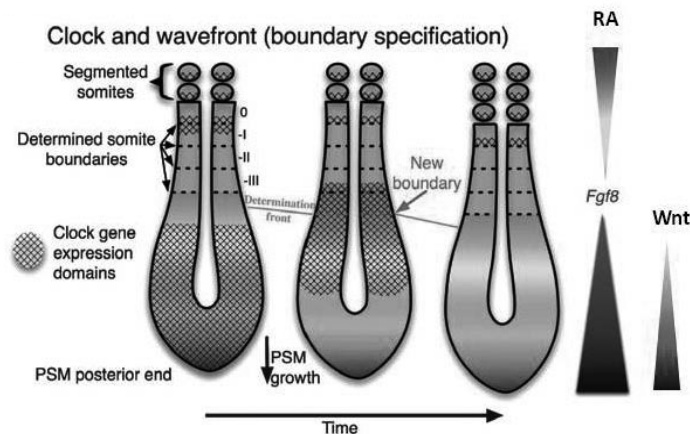


Figure 5: Somitogenesis in vertebrate involves a clock and a wavefront. Clock genes oscillate through the PSM from posterior to anterior during body elongation (grid pattern). This cyclic pattern marks within the anterior PSM the site where a somite will form. FGF, Wnt and RA gradients are believed to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

