Chapter 1

General Introduction

Part of this chapter was adapted and revised from: *Durston, A. J. and Zhu, K. (2015). Semin Cell Dev Biol 42, 86-93.*

1. Introduction

In 1802, the English poet Samuel Taylor Coleridge wrote: *"The history of man for the nine months preceding his birth would probably be far more interesting and contain events of greater moment, than all the three score and ten years that follow it."* By these words, Coleridge expressed the wonder that is probably felt by everyone of us: how do we develop from a single cell - the fertilized egg - to a complex human being? For decades, developmental biologists have been pursuing the answer to this question from many different viewpoints, which deal with different aspects of development: regional specification, cell differentiation, morphogenesis, growth and development time (Slack, 2013). Evidently, all of these aspects need to be incorporated and coordinated to get a whole picture of how development occurs. In development, one of the first tasks is to establish a body plan that describes the overall organization of the organism. This is achieved by massive reorganization of cells via a process called gastrulation.

The process of gastrulation leads to the formation of three tissue layers in the embryo: ectoderm, which gives rise to the epidermis of the skin and the nervous system; mesoderm, which forms muscles and skeleton, the notochord, connective tissues, and blood vessels etc.; and endoderm, which gives rise to the epithelial lining of the respiratory and digestive tracts and organs associated with the digestive system (Gilbert, 2010). Different organisms may use different mechanisms to achieve gastrulation, but the goal is similar: cells need to be rearranged to become located at different positions and in different tissue layers. In the frog *Xenopus*, the start of gastrulation is marked by the formation of the blastopore in the dorsal marginal zone (the dorsal lip: the dorsal-most mesoderm) (Nieuwkoop and Faber, 1994). The dorsal lip was shown, by Hans Spemann and his postdoc Hilde Mangold, to be able to induce a second axis when transplanted to the ventral side of another embryo (Spemann and Mangold, 1924), suggesting that cells in this region are able to instruct the developmental fate of other cells. This region is now referred to as the Spemann-Mangold organiser or Spemann organiser (SO). As gastrulation proceeds, future mesodermal and endodermal cells in the marginal zone continuously involute through the blastopore and move inside the embryo, where they converge towards the dorsal midline and extend towards the anterior end of the embryo under the dorsal ectoderm (convergent extension) (Ibrahim and Winklbauer, 2001; Keller and Hardin, 1987; Keller, 1986; Nieuwkoop and Florschütz, 1950; Winklbauer and Schurfeld, 1999). Meanwhile, the ectoderm spreads to enclose the whole

embryo by epiboly (Keller and Danilchik, 1988; Keller and Schoenwolf, 1977; Rozario et al., 2009; Saka and Smith, 2001). Therefore, the position of the blastopore marks the posterior end of the embryo. As gastrulation progresses, convergent extension directs cell movements toward the anterior end and elongates the embryo along the anterior-posterior (A-P) axis. The big question asked in this thesis is: how is the A-P axis formed in the developing vertebrate embryo?

1.1 A time space translation hypothesis for vertebrate axial patterning

In the fruit fly *Drosophila* (Akam, 1987; Huynh and St Johnston, 2004; Lall and Patel, 2001; Tautz, 2004), the A-P axis is related to the A-P polarity of the egg and defined by maternal-effect genes expressed in different regions of the egg, e.g. *bicoid* in the anterior region (Driever and Nusslein-volhard, 1988a; Little et al., 2011), *nanos* in the posterior region (Dahanukar and Wharton, 1996; Wang et al., 1994), and *torso* in the acron and telson (the extreme front and back ends of the fly, respectively) (Duffy and Perrimon, 1994; Li, 2005; Sprenger et al., 1989). These maternal gene products set up several concentration gradients immediately upon fertilization. For example, bicoid protein forms a high-to-low gradient along the A-P axis (Driever and Nusslein-volhard, 1988a; Driever and Nusslein-volhard, 1988b; Driever et al., 1989; Frohnhofer and Nusslein-volhard, 1986). Therefore, cells at different positions of the axis inherit different amounts of Bicoid during cellularization. This gradient information can be interpreted by cells to specify the activity of gap genes (Jackle et al., 1992; Nusslein-Volhard and Wieschaus, 1980), which in turn, regulates the expression of pair-rule and segment polarity genes. The activities of these genes eventually divide the embryo into 14 segments along the A-P axis. The developmental fate of each segment is determined by another class of genes, homeotic selector genes, which can be regulated by the protein products of the gap, pair-rule, and segment polarity genes. Therefore, the A-P axis of the fly is initially set up by unequal distribution of maternal determinants in the egg.

The vertebrate A-P axis is clearly specified by very different mechanisms. In the developing vertebrate embryo, structures along A-P axis are laid down from anterior to posterior progressively (Eyal-Giladi, 1954; Gamse and Sive, 2000; Gamse and Sive, 2001a; Nieuwkoop, 1952; Stern et al., 2006), suggesting timing is involved. In an attempt to explain how time and space are coordinated during this process, a time-space translation (TST)

mechanism has been proposed in frog to explain the patterning of the trunk (Wacker et al., 2004a)(Fig. 1). This mechanism explains how a temporal sequence of early anterior to late posterior axial gene expression generates the congruent axial spatial sequence. Briefly, a BMP (Bone morphogenetic protein) dependent timer in the gastrula's non-organiser mesoderm (NOM, the mesoderm tissue that excludes the organizer, including ventral and lateral mesoderm) undergoes sequential timed interactions with the BMP antagonistic Spemann organizer (Sasal et al., 1995; Spemann and Mangold, 1924; Zimmerman et al., 1996): a structure absents in Drosophila. The interactions occur during and after gastrulation and generate the spatial axial pattern. There is evidence that this mechanism involves Hox collinearity (Duboule and Dolle, 1989; Graham et al., 1989; Lewis, 1978) and that it requires Hox functionality (McNulty et al., 2005).

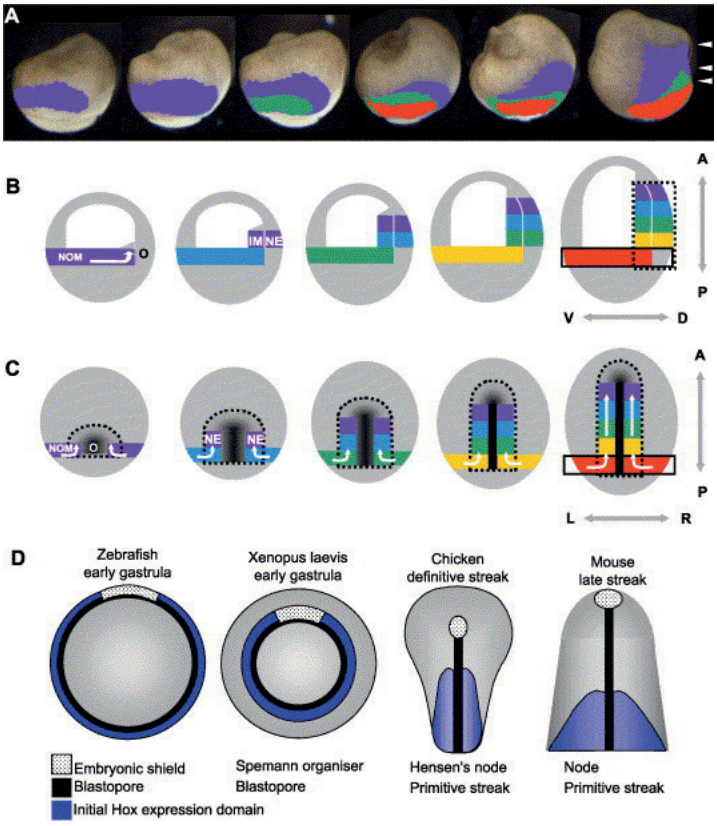

Figure 1. The time space translation hypothesis (Durston et al., 2010; Wacker et al., 2004a). (A) False colour representation of expression of an early anterior to late posterior sequence of three axial markers (Hox genes) during *Xenopus* gastrulation. Whole mount in situ hybridisation (WISH) on external lateral views of sibling embryos for *hoxd-1* (purple), *hoxc-6* (green), and *hoxb-9* (red). Digital images were analysed and selected areas labelled with respective false colour and combined in one image. Six gastrula stages (10.5, 11, 11.5, 12, 12.5 and 13) are shown in a lateral external view, anterior up and dorsal to the right. Anterior boundaries of the Hox expression at the end of gastrulation are arrowed. (B) The time space translation hypothesis. Lateral views. Time sequences of lateral views of gastrulae through gastrulation. Expression of new A–P markers is initiated in non-organiser mesoderm (NOM) at sequential times (a time sequence of more and more posterior *Hox* codes in NOM is represented by a spectral colour sequence of differently coloured horizontal bars). Non-organiser mesodermal tissue (depicted by a horizontal coloured bar which is a 2D representation of the 3D broken ring of Hox expression in the marginal zone of the wall of the embryo) moves (flows) toward the Spemann organizer by convergence and then extends anteriorly (arrow). The NOM mesoderm (IM), adjacent to the Spemann organiser involutes and its current A–P positional value (=*Hox* code) is then transferred to overlying neurectoderm (NE). While the early temporal *Hox* sequence in the non-organiser mesoderm (differently coloured horizontal bars; outlined by continuous black line in rightmost figures of B and C) is running, cohorts of new cells from this region are continually moved into the range of Spemann organiser (range represented by dashed black line) and their *Hox* code is then stabilized by an organiser signal. The temporal *Hox* sequence is thus converted into a spatial A-P pattern by continuous morphogenetic movement and stabilization of timed information by the organizer both in involuted NOM mesoderm (IM) and overlying neurectoderm (NE). The section represents a paraxial level just lateral to the organiser so the organiser is not visible. (C) The time space translation hypothesis: dorsal views. In non-organiser mesodermal (NOM) cells, the *Hox* sequence is running (differently coloured bars, solid black outline in rightmost figures). From this domain, cells are continuously moved into the influence of the Spemann organiser (dashed black outline) by convergence and extension (arrows). The AP pattern arises by sequential posterior addition of new stabilised NOM segments each expressing a different subset of *Hox* genes. A, anterior; P, posterior; V, ventral; D, dorsal; L, left; R, right. The outer neurectoderm is not shown in this figure because this section is internal, at the level of the dorsal mesoderm. (D) Time space translation occurs in all vertebrates. Schematic diagrams depicting locations of Spemann organiser, blastopore and initial *Hox* expression domain in Xenopus and orthologous structures in the zebrafish (Alexandre et al., 1996), the chick (Gaunt and Strachan, 1996) and the mouse (Deschamps et al., 1999) all shown at the beginning of gastrulation. Zebrafish and *Xenopus* are shown in vegetal views, chick and mouse are shown in dorsal views.

Hox collinearity is a term that describes the relationship between the arrangement of *Hox* genes on the chromatin and their orders of expression. In most vertebrates, Hox genes are arranged as four gene clusters (*HoxA-D*), each containing a different number of Hox genes (Figure 2). During A-P patterning, Hox genes within each cluster are expressed in a temporal sequence (early to late) that reflects their 3'to 5'arrangment (*hox1 to hox13*) (*Hox* temporal collinearity) (Dolle and Duboule, 1989; Izpisua-Belmonte et al., 1991). In the trunk part of the axis, *Hox* temporal collinearity appears to serve as a timer, which generates a congruent spatial order of *Hox* expression (anterior to posterior) along the A-P axis (*Hox* spatial collinearity) by interacting with the organiser (Durston et al., 2010; Wacker et al., 2004a). *Hox* genes thus take a higher place in the A-P patterning cascade in vertebrates than in Drosophila. Hox functionality is implicated in this mechanism because knocking out the *hox1* paralogue group not only disrupts expression of *hox1* genes but also of the whole more posterior spatially collinear *Hox* expression sequence in the early embryo's A-P axis

(McNulty et al., 2005). Evidence is emerging that this timer, which governs the neck-tail region of the axis is complemented by an earlier anti-BMP dependent time space mechanism that maps out the A-P zones in the head at the stages before gastrulation in a very similar manner (Hashiguchi and Mullins, 2013; Tucker et al., 2008). Data from Meinhardt further confirms that the vertebrate organiser generates an A-P pattern by timed application of dorsalising information (Meinhardt, 2002; Meinhardt, 2006; Meinhardt, 2015). There is thus an integral timer that regulates the development of multiple positional identities at neighbouring positions along the axis at sequential times. This time space translation mechanism clearly continues after the end of gastrulation, due to a continuation of the gastrulation process in the chordaneural hinge and other tissues in the tailbud (Gont et al., 1993). It is thus logical to think that there is an integrated BMP/anti-BMP time space translation mechanism for the whole vertebrate body axis.

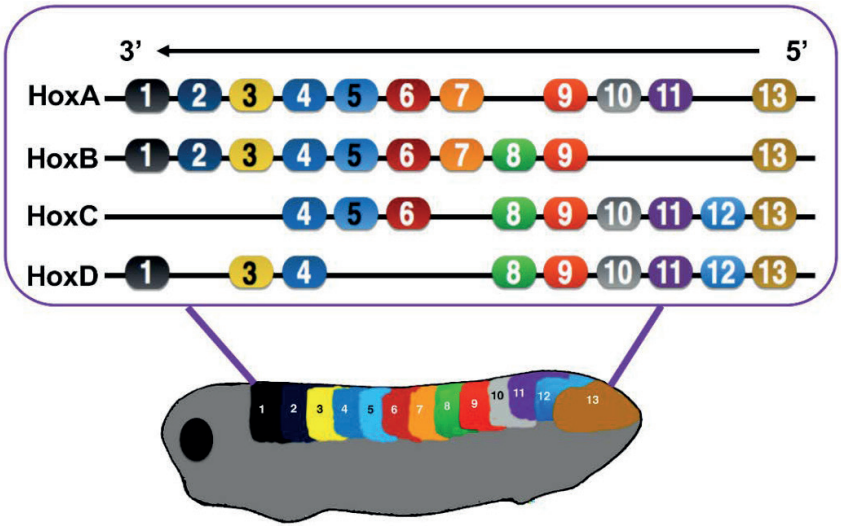

Figure 2. Chromosomal arrangement of *Hox* genes and their spatial pattern of expression along the A-P axis. Most vertebrates have four Hox clusters (HoxA, HoxB, HoxC, and HoxD). Within each cluster, *Hox* genes are arranged from 3' to 5' in a linear order (Hox1 to Hox13). This linear gene order is the same as the temporal and spatial orders of Hox gene expression during development. For example, Hox1 (HoxA1, B1 and D1) is expressed first and most anteriorly, whereas Hox 13 (HoxA13, B13, C13 and D13) is expressed last and most posteriorly.

1.2 What is the evidence that there is a timer and time space translation?

In Wacker et al's experiments (Wacker et al., 2004a; Wacker et al., 2004b), a UV ventralised BMP-rich embryo containing no organiser was challenged with an anti-BMP source at different times after the beginning of its gastrulation and the resulting embryos were analysed using in situ hybridisation. With no challenge, such an embryo simply makes a mass of ventral tissue (a 'bauchstuk', in Spemann's words) (Spemann and Mangold, 1924). With an implanted organiser (a source of anti-BMP signals), an A–P axis is regenerated. An early organiser implanted at the beginning of gastrulation induced a whole A–P body axis (Wacker et al., 2004a). Early organisers implanted at increasingly later times after the start of gastrulation in the ventralised embryo gave an increasing deletion from the anterior end of trunk part of the resulting axis, with a 6 h implantation (end of gastrulation, latest used) retaining only the most posterior part of the axis. Presumably, an implanted organiser stabilises sequentially more and more posterior positional identities from the time it is implanted. Another anti-BMP source used (corresponding with the anti-BMP signals secreted by the organiser) was blastocoel injection of the organiser anti-BMP signal: noggin protein. Again, later and later treatments were more and more posteriorising. The first treatments (at the blastula stage) induced expression of the head marker *otx2* and the anterior hindbrain marker *krox20*. The second at the beginning of gastrulation induced mainly *krox20* and the more posterior marker *hoxb4*. Later treatments than this induced a partial axis either starting with a head (earlier) or starting at a more posterior position (later). These mixed results presumably reflect the fact that noggin has multiple functions. First, it acts directly to dorsalise ectoderm, converting it to neurectoderm, thus presumably directly stabilising specific positional identities in this tissue (Jansen et al., 2007; Lamb et al., 1993). Second, it also converts NOM mesoderm to organiser mesoderm (Smith and Harland, 1992), thus permitting initiation of an axis.

These conclusions were confirmed by other studies. Dias et al. found that explanted chicken posterior primitive streak (a combination of ventral mesoderm and embryonic ectoderm) is stabilised to different positional identities by noggin applied at different times after the beginning of gastrulation (Dias et al., 2014). Early application stabilises a relatively anterior positional identity. Late application stabilises a more posterior positional identity. Mullins and colleagues also found that different positional identities are stabilised in the zebrafish embryo by differently timed application of anti-BMP (heat shock treatment of Tg

(*hsp70:chd*)) (Hashiguchi and Mullins, 2013; Tucker et al., 2008). Their studies covered the head part of the axis anterior to the trunk *Hox* gene expressing section studied by Wacker et al. (Hashiguchi and Mullins, 2013; Wacker et al., 2004a). There is thus a BMP/anti-BMP dependent TST mechanism covering the whole vertebrate A-P axis from the anterior head to the tip of the tail. The trunk timer is evidently in NOM mesoderm because addition of this tissue (but no other) to organiser mesoderm and neurectoderm in a recombinate leads to genesis of specific stable positional values in mesoderm and neurectoderm after culture. The above findings unambiguously suggest that a BMP/anti-BMP dependent TST mechanism may mediate axial patterning.

1.3 Does time-space translation involve the *Hox* genes?

Hox codes (the expression of different specific combinations of *Hox* genes) seem to be the main determinants of different A-P axial positional values in the part of the axis where the TST mechanism was discovered (the trunk) (Wacker et al., 2004a). The main axial tissues of the trunk (neurectoderm and paraxial mesoderm) each show a spatially collinear sequence of *Hox* gene expression. The above evidence argues that these are at least part of the primary axial pattern. The NOM mesoderm of the gastrula, which is the precursor of paraxial and ventral mesoderm and contains the timer, manifests a gastrula stage temporally collinear sequence of *Hox* expression. This is likely to correspond to or to be driven by the timer in the TST mechanism (Durston et al., 2010; Wacker et al., 2004a). Temporal collinearity but not spatial collinearity survives in ventralised embryos, paralleling the timer. There is no *Hox* expression in dorsalised embryos, again paralleling the effect of absence of the timer in the time space translation mechanism. Combining an organiser or a piece of dorsalised mesoderm from a dorsalised Li+ embryo with a ventralised embryo containing temporal collinearity brings back *Hox* spatial collinearity and the axial pattern as in time space translation (Wacker et al., 2004a). The function of the organiser is thus apparently to generate spatial collinearity from temporal collinearity. These features of *Hox* collinearity clearly parallel the key features of the TST mechanism, making it very likely that this is based on *Hox* collinearity. *Hox* temporal collinearity is thus likely to be needed for generating *Hox* spatial collinearity via time space translation. Knocking down the entire first *Hox* paralogue group (*hox 1*) knocks down most or all of the spatially collinear *Hox* sequence

(at least back to *hox9*), indicating that Hox1 functionality is also involved in this process (McNulty et al., 2005). A second indication of the importance of Hox function is that knocking down individual *Hox* genes in NOM mesoderm specifically prevents vertical signalling: copying of the expression of the same *Hox* genes from NOM to neurectoderm (NE) in 'wrap' recombinates, which copies the same process in vivo where vertical signalling is part of TST (Bardine et al., 2014). These findings give a clue to the mechanism of TST. Although the effects of the *hox1* knockdown on temporal collinearity are so far unknown, there seem to be strong correlations between temporally and spatially collinear *Hox* expression and the temporal and spatial phases of the TST mechanism. The *Hox* genes thus appear to be part of the core mechanism for TST. This mechanism regulates axial patterning in the trunk-tail part of the axis: the findings above argue that the mechanism acts at the level of the *Hox* genes rather than only upstream of them.

1.4 What are the roles of the organiser and NOM mesoderm in time space translation?

The organiser appears to be required to translate the time sequence in the gastrula's NOM mesoderm (putatively the gastrula's temporally collinear *Hox* expression sequence) into the spatial pattern (involving a spatially collinear *Hox* expression sequence) that appears progressively in axial mesoderm and neurectoderm during gastrulation and later. Without an organiser, in a ventralised embryo, only the NOM's transient temporally collinear *Hox* sequence is observed (Durston et al., 2010; Wacker et al., 2004a). There is no spatial axial *Hox* pattern or spatial collinearity. Without NOM mesoderm (in a dorsalised embryo), there is no *Hox* expression at all (Wacker et al., 2004a). Presumably, as successively older blocks of NOM mesoderm approach the organiser sequentially during gastrulation, due to convergence extension and involution movements, their current *Hox* identities are fixed and also copied to the overlying blocks of neurectoderm in the dorsal wall of the gastrula, this neurectoderm already having been induced from ectoderm by organiser signals. The organiser's role in this was investigated by testing how its role could be substituted. It was found that axial patterning and *Hox* spatial collinearity in neurectoderm can be achieved by tBr anti-BMP mediated dorsalisation of the ectoderm of a UV ventralised embryo only, without any need for an organiser or for dorsalisation of the embryo's mesoderm (Jansen et al., 2007). This suggests that an axial pattern can be achieved simply by organiser induction

of ectoderm to neurectoderm only, without the need for organiser action on mesoderm. However, Dias et al. have also shown recently that chicken posterior primitive streak tissue (a tissue consisting of ventral mesoderm and ectoderm) can be induced to express different stable *Hox* codes by application of the organiser anti-BMP signal noggin at different times during gastrulation, with later times giving more posterior *Hox* codes (Dias et al., 2014). This result opens the possibility that there is also a direct action of the organiser to stabilise *Hox* codes in NOM mesoderm (although this ventral streak tissue will also include embryonic ectoderm). There are still clearly unanswered questions about the role of the organiser in A-P patterning. One role of the NOM is rather clearly to transmit posterior information to neuralised ectoderm. This function involves precise copying of positional information from NOM mesoderm (vertical signalling) exactly as predicted by Mangold (Mangold, 1933). The mechanism involves very specific non-cell autonomous autoregulation of individual *Hox* genes such that their expression in NOM mesoderm is copied to neighbouring neurectoderm (Bardine et al., 2014).

1.5 What is the molecular nature of the A-P timer and of *Hox* temporal collinearity?

An obvious possibility for the timer is that this involves *Hox* temporal collinearity. The temporal sequence observed in NOM mesoderm involves a temporally collinear sequence of *Hox* expression just as the axial pattern in axial mesoderm and axial neurectoderm involves a spatially collinear axial sequence of *Hox* expression. On the other hand, it has recently been proposed that *Hox* temporal and spatial collinearity are not connected (Noordermeer et al., 2014; Tschopp et al., 2009). The temporal *Hox* sequence observed during gastrulation is the classical example of *Hox* temporal collinearity. It has been proposed that this and the other examples of *Hox* temporal collinearity depend on progressive 3' to 5' opening of the Hox clusters for transcription (Chambeyron et al., 2005; Noordermeer et al., 2014). There is evidence that this occurs but it is not the whole story for *Hox* temporal collinearity and timing during gastrulation. Hox temporal collinearity needs synchronisation of the structurally different *Hox* clusters which may involve trans interactions within cells and intercellular interactions between different cells in the synchronized mesoderm of the gastrula (Durston, 2012; Durston et al., 2011). The *Hox* temporal sequence is also part of a larger A-P axial sequence including non *Hox* homeobox

genes in the head (Hashiguchi and Mullins, 2013), again arguing against *Hox* cluster opening as a global mechanism. Notably, Hox1 functionality is clearly involved in generating the early spatially collinear axial *Hox* sequence (McNulty et al., 2005). What is possible is that gastrula temporal collinearity involves collinear interactions among *Hox* genes. Two interactions could putatively play a role. Posterior dominance, where posterior *Hox* genes inhibit expression and function of more anterior ones (Durston, 2012; Durston et al., 2011; Hooiveld et al., 1999; Lewis, 1978; Woltering and Durston, 2008). Posterior induction, where anterior Hox genes induce expression of more posterior ones (Durston et al., 2011; Hooiveld et al., 1999; McNulty et al., 2005). They are known to occur in the early vertebrate embryo, but there is no evidence so far that they are the basis of *Hox* temporal collinearity or of the A-P patterning timer.

1.6 What is upstream? Does *Hox* collinearity drive the axial patterning clock or is it driven by it?

The above sections argue that time space translation occurs partly at the level of the *Hox* genes. There may also be upstream inputs involved in this process. It is possible that *Hox* collinearity, based on collinear interactions between the Hox genes (Hooiveld et al., 1999), is the basis for time space translation. There are also other suggestions. It has been proposed for the anterior timer in the head that BMP regulated Smads interact at the protein phosphorylation level with classical A-P patterning pathways: FGFs, Wnts and retinoids (Hashiguchi and Mullins, 2013). There is also evidence that the somitogenesis clock may be involved in regulating Hox temporal collinearity (Cordes et al., 2004; Durston, 2015; Peres et al., 2006). Upstream regulators of the timed anterior early axial patterning genes have also been identified in the neurectoderm (Gamse and Sive, 2001b). Besides Hashiguchi and Mullins' proposal of the mechanism for A-P patterning, there are also previous proposals that have involved actions of the Wnt, retinoid and FGF signalling pathways (Durston, 2015). An important question is: how do these pathways act?

A-P patterning is clearly a long and complex process. Perhaps these signalling pathways are involved in another part of the process, either later or concurrently in a parallel mechanism. However, they could also presumably be involved in time space translation. They may act at 'decision points', in time and spatially, on the axis, where cells switch their regulation by

different pathways and factors anterior to and before as opposed to posterior to and after their decision (Durston, 2015).

2. Outline and aim of this thesis

The mechanism underlying origin and patterning of the vertebrate anterior-posterior (A-P) axis is a very old long-standing problem that is still unsolved. In *Drosophila*, the mechanism has to do with unequal distribution of maternal determinates, which regulate genes involved in patterning and segmenting the embryo along the A-P axis (Akam, 1987; Huynh and St Johnston, 2004; Lall and Patel, 2001; Tautz, 2004). Less is known about how vertebrates make their A-P axis. It has been noticed for a long time that there is an age structure along the vertebrate A-P axis: more anterior tissues are older than more posterior ones, suggesting that timing is involved in A-P axis formation. Therefore, a solution to this problem in vertebrates must encompass two important issues: 1) how is timing regulated during A-P axis formation? 2) how are time and space coordinated?

To address both issues, a time-space translation mechanism was proposed based on evidence presented in above sections. This mechanism involves a BMP dependent axial timer. Sequential time/position values from this timer are stabilized by anti-BMP signals from the Spemann organiser during gastrulation, thus progressively generating an axial pattern. The timer in this mechanism may involve early *Hox* temporal collinearity and the organiser may control sequential transitions from *Hox* temporal collinearity to expression zones in a spatially collinear *Hox* sequence, leading to patterning of the trunk part of the axis. **Chapter 2 and 3** aim to understand the driving force of the *Hox* timer. To this end, in **Chapter 2**, we carry out gain-of-function studies in wild-type and *noggin*-injected (*Hox* repressive) embryos. The results of these studies reveal two important Hox-Hox interactions: Posterior induction (induction of posterior *Hox* genes by anterior ones) and posterior dominance (repression of anterior *Hox* genes by posterior ones). We demonstrate that these Hox-Hox interactions are important for vertebrate axial patterning. This conclusion is tested further in **Chapter 3** by Hoxc6 loss-of-function. Preliminary results from our group showed that Hoxc6 loss-of-function produced a severe defect in A-P axis formation. We therefore knock down *hoxc6* by morpholino injection. This truncates the body axis at the neck-thorax

boundary and cuts off the *Hox* gene sequence at the *Hox6* level. These findings further support a role for Hox-Hox interactions in vertebrate axial patterning.

This mechanism may connect to a very similar TST mechanism in the head part of the axis. It has been shown that anterior brain markers: *six3* (Kobayashi et al., 1998)*, otx2* (Li et al., 1994; Mori et al., 1994) and *gbx1* (Rhinn et al., 2003) have their expression domains sequentially expanded by early anti-BMP signals, in the blastula and early gastrula stages before the *Hox* timer starts (Hashiguchi and Mullins, 2013). This mechanism and the *Hox* TST mechanism seem to be parts of the same continuum, as the sequence is continued by *hox1* (Hashiguchi and Mullins, 2013). If a BMP-dependent timer is also running in the head, according to the TST hypothesis, it should be sequentially stopped by timed anti-BMP treatment in ventralised embryos, which contain only BMP signals. To test this, in **Chapter 4**, we produce ventralised embryos by overexpression of *bmp4* (Dale et al., 1992; Jones et al., 1992; Schmidt et al., 1995), which gives rise to a blob of tissue with no axis. Timed anti-BMP signals are introduced into these embryos from blastula to early gastrula stage by using a *smad6GR* construct. In this experimental setup, the expression of the anterior brain markers (*six3, otx2, gbx2*) and *Hox* genes (e.g. *hoxd1*) are sequentially stopped. Since cement gland is part of the extreme anterior domain (EAD) (Dickinson and Sive, 2007) and is the most anterior tissue in the frog embryo (Sive et al., 1989), we also examine the expression of its maker gene *xcg-1* (Gammill and Sive, 2000). However, the involvement of *xcg-1* in the sequence is not clear, since in the time window we examine gene expression, only one embryo (out of 17) shows *xcg-1* expression at stage 8.

The anterior gene sequence shares some similarities with the Hox gene sequence. First, it parallels the A-P axis of the head; second, the sequence can be sequentially arrested by timed anti-BMP treatment. Third, they are also homeobox genes. Based on these similarities, it is logical to postulate that the anterior genes are sequentially expressed and constitute a timing mechanism in the head part of the axis. We therefore examine the expression of these genes in wild type embryos at different stages. However, they are not sequentially expressed, suggesting that an upstream timing mechanism may be involved in regulating these genes. **Chapter 5** is a conceptual analysis on the patterning of the head and trunk by time-space translation and how they are connected to make the whole axis. In the memory of D'Arcy Wentworth Thompson, the author of the famous book "On Growth and Form" (Thompson, 1917), in Chapter 5 we attempt to theoretically elucidate the developmental principle

underlying A-P patterning in vertebrate. In a nutshell, this thesis aims to understand how temporal information is translated into spatial patterns during A-P patterning and how this may involve the opposing actions of BMP and anti-BMP.

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