Directing the Development of Posterior Neural Fates: The Role of FGF, Cdx, and hox genes

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DIRECTING THE DEVELOPMENT OF POSTERIOR NEURAL FATES: THE ROLE OF FGF, CDX, AND HOX GENES

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Understanding how the anatomical complexity of the nervous system arises is key to understanding the function of the nervous system. Neural development is a complex process consisting of multiple discrete steps regulated and coordinated by signaling events. Surprisingly, a rather small number of signals are implicated in regulating these vastly different neural developmental processes. A striking example is the case of the signaling molecule Fibroblast Growth Factor, or FGF, which regulates the induction of neural tissues from prospective ectoderm cells, regionalizes the neural tissue into forebrain, midbrain, hindbrain, and spinal cord, and provides discrete anterior-posterior (A-P) identity to hindbrain and spinal neurons in a manner that is ordered and patterned. This dissertation focuses on understanding the molecular switches that allow FGF to regionalize and then pattern the most posterior regions of the neural tissue, the hindbrain and spinal cord. Using the zebrafish as an experimental model system to increase or eliminate gene and signaling pathway activities, data presented here shows that the regionalization and patterning functions of FGF are regulated by the transcription factor Cdx4. Based on this
evidence, a model is proposed in which feedback cooperation between Cdx4 and FGF signals are essential for the proper spatial and temporal regulation of a large family of A-P patterning genes, the hox family of transcription factors. In this way, emergence of the hindbrain and spinal cord regions and the subsequent A-P patterning is dependent on the sequential activation of different classes of transcription factors that can change the response of cells to those very same signals in a unidirectional way. This sequential and unidirectional activation of genes by signaling regulators may be a common mechanism to drive development during embryogenesis.
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Chapter 1: Introduction

The nervous system is the primary communication network of the organism whose origin can be traced back to the development of the early embryo.

The main information processing center of vertebrates is the central nervous system (CNS), which is composed of the centrally located brain and spinal cord, and functions to send input and receive output signals to and from organs and tissues (Lumsden and Krumlauf, 1996). The CNS is able to communicate with every organ and tissue via complex innervations located throughout the organism. These innervations are collectively known as the peripheral nervous system (PNS). Thus, the peripheral nervous system serves as a conduit of information from the environment and internal organs to the processing information center that is the central nervous system.

The organization of the CNS is critical to its function. Architecturally, the CNS is broadly divided from anterior to posterior into the brain, which is further subdivided into the forebrain, midbrain, and hindbrain, and a posterior spinal cord (Lumsden and Krumlauf, 1996). The forebrain will become the telencephalon, hypothalamus, thalamus and diencephalon, which will process all internal and external sensory information and regulate higher intellectual and homeostatic operations (Beccari et al., 2013; Lupo et al., 2014). The midbrain, which becomes part of the brainstem, functions to process visual and auditory sensory cues, as well as coordinate visual cues with motor function (Moreno-Bravo et al., 2014).
The other part of the brainstem originates from the hindbrain and functions to regulate key autonomic functions like respiration, circulation, and force and range of motor functions (Moens and Prince, 2002). Finally, the spinal cord functions to distribute sensory inputs and motor outputs to the brain and target organs, as well as process some autonomic functions in extremities, such as reflexes (Tanabe and Jessell, 1996). Each specific region of the nervous system contributes to the overall functionality of the CNS, therefore, understanding how the CNS is structurally established has, and will continue to, provide key insight into how the nervous system functions.

The complex organization of the adult CNS can be traced back to its simple organization in embryos. CNS development starts with the formation of a single epithelial group of cells, located dorsomedially in the pre-gastrula embryo, which progressively becomes specified into different regions based on the constituting cell’s position along three major embryonic axes (Ghysen, 2003; Stern, 2006). The first phase of neural development is known as neural induction, when ectodermal cells fated to become dermis are specified to acquire a neural fate (Stern, 2006). The process of neural induction is under strict control of several molecular signals that include inhibitors of neural fate, such as bone morphogenic proteins (BMP) expressed in the ectoderm, and inhibitors of BMP activity, such as Chordin, Noggin and Follistatin, secreted from a dorsally located structure known as the Organizer (Spemann and Mangold, 2001; Wilson and Hemmati-Brivanlou, 1995). In addition, neural induction also needs the positive effector fibroblast growth factors or FGFs (Kengaku and Okamoto, 1995; Lamb
and Harland, 1995). Once the epithelial sheet has acquired neural characteristics, it undergoes a series of morphogenetic movements that culminate in its rolling into a tube, a process known as neurulation (Colas and Schoenwolf, 2001). Highlights of neurulation, including the apical constriction of the base of cells as well as convergent extension movements, must be coordinated to bend the neural plate into a grooved canal and then the closing of this groove to form a tube (Davidson and Keller, 1999; Smith and Schoenwolf, 1997; Ybot-Gonzalez et al., 2007). Simultaneously, crude patterning events along the Anterior-Posterior (A-P) axis of the neural tube start subdividing it into its four main anatomical regions (Vaage, 1969). This regionalization process is again under the global control of several signaling factors: Wnt-inhibitors anteriorly and Wnts and FGFs posteriorly (Elkouby et al., 2010; Isaacs et al., 1994). In addition, localized expression of several other factors also helps in this regionalization. For example, FGF in the midbrain/hindbrain boundary (Crossley et al., 1996; Liu et al., 1999) and RA at the hindbrain-spinal cord transition (Gavalas and Krumlauf, 2000). Finally, these broad territories are further subdivided along the A-P and Dorsal-Ventral (D-V) axes to establish all of the distinct neuronal cell populations of the CNS (Lumsden and Krumlauf, 1996). The coherent acquisition of specific identities by cells is known as patterning and requires, in the A-P dimension, the activity of the transcription factors Hox and Cdx (Copf et al., 2004; Duboule and Dolle, 1989). These multiple discrete steps act coordinately to gradually generate, from a simple neuroepithelia, the complex organ that is the CNS.
Although the molecular signals driving each discrete step have been identified, many of them are used recurrently to drive multiple processes, and it is unclear how the same signal can elicit different responses in different regions. For example, FGF is essential for the specification of cells to neural identity with anterior characteristics (Hongo et al., 1999; Kengaku and Okamoto, 1995), and is also essential later on for the specification of these neural cells to more posterior identities (Lamb and Harland, 1995). What is the molecular context by which the same signal elicits different responses in different regions using the same transduction pathway remains unknown. More importantly, it is still unknown how these signals cooperate to govern the vast array of processes driving neural development in coordination. Here, first I analyze the expression and regulation of a vast family of transcription factors required for posterior neural identity, the hox genes, and then, I analyze the signaling and transcription factor networks that regulate hox transcription. From these studies I conclude that hox gene regulation and posterior neural patterning is under the global control of the transcription factor Cdx4, which cooperates with FGF signals in a positive feedback loop to maintain hox transcription and cell identities throughout early nervous system development.

**Nieuwkoop’s Activation-Transformation model of CNS development**

A generally accepted model to explain the early induction and subsequent patterning of the CNS was proposed in 1952 by Pieter Nieuwkoop (Nieuwkoop,
In this model, two separate signaling events were proposed to drive the early induction, or “activation” of ectodermal cells to acquire anterior neural fates, and the subsequent specification or “transformation” of these anterior neural fates to more posterior neural identities (Fig. 1.1). In transplantation experiments using amphibian embryos, naive ectoderm explants obtained from different axial position of early donor embryos were attached at different A-P positions of host’s neural plate. Transplanted tissue, irrespective of their site of origin, developed the identity of the region in which they were attached. Furthermore, they also developed the identities of more posterior regions, and never the identities of more anterior regions (Nieuwkoop, 1952a, b). For example, a tissue fragment attached to the head region, developed head, trunk and tail identities, whereas a tissue fragment attached to the trunk, only developed trunk and tail identities (Nieuwkoop, 1952a, b). These results provided support for signaling events that specify A-P positional information to tissues so cells know where they are located: head, trunk or tail. This signal, or signals, is different from the neural inducing signals provided by the region known as the dorsal organizer first described by Hans Spemann (Spemann and Mangold, 1924). Spemann’s first signaling event and Nieuwkoop’s second signaling event are different because Spemann’s signals induce ectoderm to become neural tissue with anterior identities, whereas Nieuwkoop’s transformation signals can only change the identity of already specified neural cells to progressively more posterior fates. Over the last 70 years one of the key topics of research in developmental neurobiology has been
discerning the molecular signals that underlie Nieuwkoop’s model, the induction
and subsequent patterning of neural tissues.

**Neural Induction**

Neural induction can be characterized at the cellular and molecular level in
multiple steps (Wilson and Edlund, 2001). In the first step, cells from the
ectodermal germ layer are instructed or specified to take on a neural fate early in
gastrulation (Davidson and Keller, 1999; Schoenwolf, 1988; Shih and Fraser,
1996; Streit et al., 2000). This fate is labile, and cells can still follow an
ectodermal mode of development. In a second step, cells become committed to
a neural fate, which cannot be altered regardless of their environment (Wilson
and Edlund, 2001). Cells that are committed to a neural fate acquire a columnar
epithelial organization and form a thickened epithelium, the neural plate (Appel,
2000; Bouwmeester, 2001; Colas and Schoenwolf, 2001; Papalopulu and
Kintner, 1992). Additional support for the sequential specification and
commitment of cells to a neural fate comes from a series of studies in chicken
(Wilson et al., 2000). In these studies, only prospective neural plates explants
from embryos at mid-gastrulation, but not late gastrulation, could be instructed to
revert back to their original ectodermal fate by the addition of BMP4 (Wilson et
al., 2000), supporting the idea that neural commitment is a late event in a series
of inductive events driving neural tissue development.
Neural induction requires a number of molecular signaling events. The first signaling candidates shown to play a role in the neural inductive process were identified in *Xenopus* studies. A puzzling experiment in the early 1990’s showed that dissociated ectodermal cells that normally give rise to ectoderm, when grown at low density in culture, acquire a neural fate (Godsave and Slack, 1991; Grunz and Tacke, 1989). Given that these cells were not exposed to any signaling factor, it was proposed that ectodermal cells had a natural propensity to develop as neural tissue. Support of this idea came from similar experiments in which treatment of ectodermal explants with an inhibitor of Activin, a transforming growth factor beta (TGF beta) family member, caused the explant cells to change their fate from ectodermal to neural (Hemmati-Brivanlou and Melton, 1992). Activin is not present in the early embryo, but closely related molecules, the BMPs, are present (Reversade et al., 2005; Wilson and Hemmati-Brivanlou, 1995). Importantly, the organizer was found to secrete the Activin/BMP inhibitors Chordin, Noggin and Follistatin (Hemmati-Brivanlou et al., 1994; Lamb et al., 1993; Sasai et al., 1995), which could block BMPs binding to their receptors to drive neural population development (Piccolo et al., 1996; Yamashita and Miyazono, 1999; Zimmerman et al., 1996). Together these results supported a model in which the default fate of ectodermal cells is to become neural, but autocrine BMP signaling directs these ectodermal cells to take on epidermal fates. Thus, in order to establish a neural population, a negative cue is required to block ectodermal signaling, allowing neural fates to become manifest.
Evidence from several species support more complex events than those described in the default model of neural induction. In *Xenopus*, some reports indicated that inhibition of BMPs by Chordin and Noggin was not sufficient to induce neural fates in animal cap explants (Launay et al., 1996; Sasai et al., 1996). In the chick, over-expression of BMPs, or the BMP antagonist *Chordin*, did not alter the size or position of the neural plate (Streit et al., 1998), but was required later in development to stabilize the fates of cells already induced to become neural (Linker and Stern, 2004), and in zebrafish, BMP over expression did not abrogate neural tissue (Koshida et al., 2002). Similarly in mouse, mutants lacking various BMP antagonists still developed a neural tube (Bachiller et al., 2000; McMahon et al., 1998; Mukhopadhyay et al., 2001). Together these results provide key evidence that BMP, while necessary for stabilizing neural fates, was not sufficient for neural induction.

In the search of a neural inducing signal, FGF soon emerged as another factor required for neural specification. Using dissociated *Xenopus* embryos grown in culture, it was shown that FGF was capable of inducing various anterior and posterior neural markers (Hongo et al., 1999; Kengaku and Okamoto, 1993, 1995; Lamb and Harland, 1995). Because these treatments did not induce mesoderm development in these cells, FGF was proposed as a direct neural inducer (Lamb and Harland, 1995). Equivalent results have subsequently been obtained in zebrafish (Kudoh et al., 2004). Importantly, experiments in chicken showed that FGF coated beads can induce epiblast cells to acquire neuroectodermal properties before the appearance of an organizer, and before
the expression of anti-BMP genes (Streit et al., 2000). Furthermore, FGF could cause the downregulation of BMPs in epiblast cells (Wilson et al., 2000; Wilson et al., 2001). In reciprocal loss of function experiments, when FGF signaling was blocked pharmacologically, BMP signaling was not suppressed and the cells became epidermal. Together, these results have led to the idea that FGF directly induces a labile neural fate on ectodermal cells that subsequently needs to be stabilized by anti-BMP activity. FGF also plays an indirect role in neural fate stabilization because it can down-regulates the activity of the BMP pathway (Delaune et al., 2005; Munoz-Sanjuan and Brivanlou, 2002; Wilson et al., 2000). Thus, working in cooperation, FGF instructs and BMP stabilizes neural cell fate (Streit et al., 2000).

**Regionalization of the central nervous system**

Regionalization is the process of establishing the segmental identity of the vertebrate CNS. The earliest morphological landmarks of regionalization are the three main vesicles of the brain, the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain), as well as the posterior region devoid of ventricles known as the spinal cord (Vaage, 1969). These early ventricles are delineated by a series of ring-like constrictions around the developing neural tube (Rubenstein et al., 1998). Further regionalization becomes evident with the further division of the forebrain into the telencephalon
and diencephalon, along with the segmentation of the hindbrain to establish eight rhombomeres (Lumsden and Krumlauf, 1996).

Regionalization of the nervous system begins before neurulation and is critically dependent on signals originating in tissue outside of the developing CNS. Classic transplantation experiments have shown that the neuroectoderm develops following the regional identities of surrounding mesoderm. In *Xenopus*, transplanting mesoderm underneath the neural plate changes the regional identity of the neural plate to the regional identity of the transplanted mesoderm (Mangold, 1933). For example, posterior mesoderm tissue transplanted next to anterior neural plate will transform the identity of the neural plate from anterior to posterior. In reciprocal experiments, transplantation of a naïve neural plate tissue next to mesoderm, at different axial levels, changes the developmental potential of the neural explant to that of the host tissues (Nieuwkoop and Nigtevecht, 1954). So, naïve neural plate transplanted next to head mesoderm will induce the transplant to become forebrain tissue, whereas if transplanted to the tail region, the explant will develop as forebrain, midbrain, hindbrain, and spinal cord (Nieuwkoop and Nigtevecht, 1954). Together, these results suggest that the underlying mesoderm provides instructive A-P signals to the neuroectoderm. Although these early signals provide crude regional identity to the developing CNS, this information is labile, as cells are not yet committed to attain a specific regional fate; at later stages, cells can be transplanted along the A-P axis of the neural plate, attaining the fate of the region they are transplanted to (Schoenwolf, 1992). Thus, as in the case with neural induction, the processes
of neural regionalization depend on the induction and later stabilization of fates. Because change in identity is under the influence of the mesoderm, extracellular signaling events must be directing these transformation events.

Regional specification takes place before any overt changes in tissue morphology, and is driven by the activity of transcription factors working as "master regulators" specifically expressed in each one of the neural territories. The first insight into the genes specifying regional identities came from isolation of transcription factors required in *Drosophila* for segment regionalization (Akam, 1987), and later found to have homologues in vertebrates (Simeone et al., 1992). Homologues of *Drosophila*’s orthodenticle gene expressed in the fly’s head and brain, the vertebrate *otx* gene, was found to be expressed in the mouse forebrain and midbrain during gastrulation where it plays an essential role in the specification of these regions (Acampora et al., 1995; Ang et al., 1996; Simeone et al., 1993). A second gene, the mouse *gbx* gene, was shown to be expressed early in development and be essential for midbrain and anterior hindbrain specification (Wassarman et al., 1997). Loss of Otx in the forebrain or Gbx in the midbrain/anterior hindbrain, transform the identity of the tissue where they are expressed to the identity of adjacent (Broccoli et al., 1999; Millet et al., 1999; Rhinn et al., 2005; Wassarman et al., 1997). Evidence for the active specification of spinal cord did not come until many years later with the discovery that Cdx transcription factors, a family of genes expressed early in gastrulation, are necessary and sufficient for spinal cord specification (Skromne et al., 2007). In the zebrafish when *cdx1a* and *cdx4* are absent, spinal cord is lost at the
expense of hindbrain. Conversely, ectopic expression of \textit{cdx4} in the hindbrain disrupts hindbrain development and promotes the development of spinal cord tissue (Skromne et al., 2007). Together these results suggest that Otx in the forebrain and midbrain, Gbx in the midbrain, and Cdx in the spinal cord direct the regional specification of broad neural regions of the vertebrate nervous system. These transcription factors act as “master regulators”, as they are high in the regulatory cascade controlling the identity of cells in each region.

\textbf{Patterning of the neuroectoderm}

During the broad subdivision of the CNS into the four large territories, cells in the most posterior territories, the hindbrain and the spinal cord, are further assigned local identities that become manifest as metamerically, although not necessarily segmented, units. The hindbrain is distinguished from the most posterior spinal cord by the formation of seven/eight segments or rhombomeres (Lumsden and Keynes, 1989; Trevarrow et al., 1990; Vaage, 1969), each hosting specific populations of motor and sensory neurons (Guthrie, 2007; Hanneman et al., 1988; Lumsden and Krumlauf, 1996). In contrast, the spinal cord does not have morphological segments, but has metamerically arranged inter- and motor-neurons (Francius et al., 2013; Landmesser, 2001). These arrangements are dependent on mesoderm-derived signals interacting with spinal cord progenitors (Ensini et al., 1998; Guidato et al., 2003; Lewis and Eisen, 2004). Significantly, lateral motor columns are not metameric, but instead, only exist at the level of the fore- and hind-limbs (Landmesser, 1978a, b). In both the segmented hindbrain
and unsegmented spinal cord, cell populations are assigned specific A-P identities that correspond to their A-P position along the embryos. This genetically encoded information is critical for their function.

Assignment of A-P identities is under the control of hox genes. Hox genes were first identified in *Drosophila* as series of mutations in the *Antennapedia/Bithorax* gene complex, collectively known as the homeotic gene cluster (*HOM-C*), which produced embryos with various segmental A-P transformations (Lewis, 1978). When all three genes of the *Bithorax* portion of the *HOM-C* complex are absent the fly embryo only develops from the head back to the second thoracic segment and the remainder of the posterior segments are homeotically transformed to become identical to the second thoracic segment (Lewis, 1978). Conversely, over-expression of *Antennapedia* in *Drosophila* leads to the lack of head involution or the transformation of head structures to thoracic structures, such as ectopic limbs developing in the place of antenna (Hazelrigg and Kaufman, 1983; Schneuwly et al., 1987). The *HOM-C* gene cluster vertebrate homologs are highly conserved (Duboule and Dolle, 1989; Gaunt, 1988; Graham et al., 1989), but have undergone a series of duplication events in the vertebrate: the split *HOM-C* complex in *Drosophila* equates to four complexes in mammals (Duboule and Dolle, 1989; Gaunt, 1988; Graham et al., 1989), and eight to nine complexes in teleost fish (Amores et al., 1998). Because of these duplication events, hox related by descent can be identified in the clusters, that is, they are paralogs. These paralogs share similar expression domains. For example, paralog group 3 genes are expressed at similar axial levels, the
posterior hindbrain, regardless of which cluster they are on (Greer et al., 2000). Genetic manipulations have shown hox genes to be critical for conferring positional information to cells along the embryo’s A-P axis. This is most clearly shown by loss of function experiments in which hox gene elimination results in specific homeotic transformation specifically at the level where the hox gene is most anteriorly expressed. In mouse, loss of hoxa1 results in the severe reduction (Carpenter et al., 1993) or absence (Mark et al., 1993) of rhombomere five and associated cranial motor neurons VII & VIII. In contrast, mouse mutants for hoxb1 fail to form rhombomere 4 and the associated facial nerve VI (Studer et al., 1996). These phenotypes are exacerbated by the elimination of both hoxa1 and hoxb1 (Carpenter et al., 1993; Gavalas et al., 1998). Remarkably, similar phenotypes were observed in zebrafish when the homologous genes, hoxb1a and hoxb1b were eliminated using antisense morpholino approaches, which suggests evolutionary conservation of the function of these duplicate genes in zebrafish and mouse (McClintock et al., 2002). Additional support comes from gain of function experiments. In several systems, overexpression of a posterior gene in a more anterior region transforms the identity of the anterior region to that of a posterior one. For example, in mouse, overexpression of the rhombomere 4 gene hoxa1 in rhombomere 2 transforms the nerves in r2 to acquire morphology of r4 nerves (Zhang et al., 1994). Similar results were observed in chicken, where ectopic expression of rhombomere 4 gene hoxb1 in rhombomere 1, also transformed the identity of cells in rhombomere 1 to rhombomere 4 identities (Jungbluth et al., 1999). Equivalent experiments in the
spinal cord support similar functions of hox genes in this neural region (Philippidou and Dasen, 2013). For example, disruption of the anterior spinal cord genes hoxa5 & hoxc5 results in phrenic motor column disorganization (Philippidou et al., 2012). Similar to what is observed in the hindbrain, disruption of any of the spinal cord genes hox6 to hox13 causes disruptions in motor column formation in the region where the gene is more anteriorly expressed (Economides et al., 2003; Holstege et al., 2008; Hostikka et al., 2009; Jung et al., 2010; Lacombe et al., 2013; Lin and Carpenter, 2003; Tiret et al., 1998; van den Akker et al., 1999; Vermot et al., 2005; Wahba et al., 2001; Wu et al., 2008).

Together, these results suggest that the identity of cells along the A-P axis is regulated by hox gene activity, and that the patterned expression of these genes is what is important for conferring ordered positional information to cells.

Given the powerful activity that hox genes have in conferring A-P identity to cells, multiple, tightly regulated controls exist to deploy their expression. The observation that hox gene clustering is evolutionarily conserved across metazoan, suggested that this organization was important for their regulation (Kmita and Duboule, 2003; Krumlauf, 1994; Philippidou and Dasen, 2013). Expression analysis studies showed nested overlapping expression patterns of the hox genes in the CNS and mesoderm, in an anterior-to-posterior direction that is collinear with the 3'-to-5' organization of genes in a cluster (Duboule, 1992; Kessel and Gruss, 1990; Keynes and Krumlauf, 1994; Krumlauf, 1992; McGinnis and Krumlauf, 1992). Hox genes are organized in a 5' to 3' direction in the clusters, with more 3' genes expressed in more anterior regions of the
embryo than 5’ genes, which are expressed more posteriorly (spatial collinearity) (Duboule, 1992; Kessel and Gruss, 1990; Keynes and Krumlauf, 1994; Krumlauf, 1992; McGinnis and Krumlauf, 1992). Significantly, 3’ anterior genes are also expressed much earlier in development than more 5’ posterior genes (temporal collinearity) (Duboule, 1992; Kessel and Gruss, 1990; Keynes and Krumlauf, 1994; Krumlauf, 1992; McGinnis and Krumlauf, 1992). The importance of collinearity in specifying hox expression domains come from deletion studies. Systematic deletion of hox genes within the hoxd cluster of mouse showed elegantly that the position of the gene within the cluster is critical for their spatiotemporal activation. Deletions eliminating one, two or even three genes in the cluster resulted in the earlier and more anterior expression of more posterior genes (Tarchini and Duboule, 2006). This, and other similar experiments (Duboule and Morata, 1994; Durston et al., 2011), support the claim that the evolutionary conservation of hox cluster structure in most animals is essential for proper deployment of hox transcription in time and space.

Regulation of hox transcription and CNS patterning: the roles of Retinoic Acid and FGF

Hox transcription has been shown to be under the regulation of several signaling factors, including Retinoic Acid (RA) and FGF. RA plays an essential function in regulating the transcription of hox genes expressed in the hindbrain and anterior spinal cord. This regulation is mediated through RA binding to its nuclear receptors, which then bind to Retinoic Acid Response Elements located
in various enhancer regions of the hox genes (Ahn et al., 2014; Dupe et al., 1997; Mainguy et al., 2003; Nolte et al., 2003; Studer et al., 1994). This binding initiates hox transcription and establishes the proper anterior limit of expression (Ahn et al., 2014; Dupe et al., 1997; Gould et al., 1998; Mainguy et al., 2003; Nolte et al., 2003; Nolte et al., 2013; Studer et al., 1998; Studer et al., 1994). In contrast, FGF primarily regulates the expression of trunk and tail hox genes. When dissociated Xenopus ectodermal cells were ectopically treated with FGF posterior hox genes were induced, but not anterior hox genes (Lamb and Harland, 1995; Pownall et al., 1996). Similarly, when chicken embryos were ectopically treated with FGF, no changes were observed in the neural expression of hoxb4, yet the expression of hoxb9 expanded anteriorly (Bel-Vialar et al., 2002). More convincingly, loss of function studies in the Xenopus showed that in the absence of FGF signaling activity, posterior hox gene expression was lost (Pownall et al., 1998). Although these studies support a connection between FGF activity and posterior hox gene expression, a molecular link between FGF pathway activation and hox genes enhancer regions is still missing.

Additional regulatory roles of FGF in CNS development: FGF role in CNS regionalization

FGF is a multi-faceted regulator of CNS development, and in addition to its roles as a neural inducer and hox gene regulation, FGF signaling has been implicated as a candidate for Nieuwkoop's transformation signal. As previously discussed, FGF is an inducer of posterior hox gene expression. Significantly,
however, was the observation that hox gene induction was dependent on the length of time and concentration that tissues were exposed to FGF. Xenopus animal caps treated as whole or dissociated cells with different amounts and, for different periods, with bFGF resulted in the progressive posteriorization of the neural tissue (Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Slack and Isaacs, 1994). Equivalent studies in the chick, mouse, and zebrafish have led to similar conclusions (Davidson et al., 2000; Koshida et al., 2002; Storey et al., 1998). Thus, FGF functions in the induction, regionalization and patterning of neural tissues. To date, it is still unknown what mechanisms allow FGF, to almost simultaneously, regulate the three distinct processes of neural induction, regionalization and patterning.

**Cdx genes and CNS regionalization and patterning**

One family of transcription factors that are likely candidates to coordinate neural developmental progression from regionalization to patterning is Cdx. In addition to Cdx role in specifying the spinal cord (Skromne et al., 2007), these genes are also key in patterning axial tissues through the regulation of hox gene transcription (Chawengsaksophak et al., 2004; Chawengsaksophak et al., 1997; Davidson et al., 2003; Faas and Isaacs, 2009; Kinkel et al., 2008; Shimizu et al., 2005; Subramanian et al., 1995; van den Akker et al., 2002; Wingert et al., 2007). In mouse, loss of function studies support a role of Cdx family members in specification and patterning of trunk and tail regions. Mice homozygous for Cdx1
mutations develop vertebrae that are homeotically transformed to more anterior regions, that is, posterior structures acquire the identity of anterior regions (Subramanian et al., 1995). These transformations correlate with posterior shifts in hox gene expression. Similar changes in hox expression and skeletal structure transformations are observed in Cdx2 heterozygous mutant mice (homozygous mutant mice die pre-implantation due to defects in extra-embryonic tissue) (Chawengsaksophak et al., 2004). Importantly, Cdx1 homozygous mutant/ Cdx2 heterozygous mutant compound mice display even more severe trunk and tail homeotic transformations and changes in hox gene expression than single mutations alone (Chawengsaksophak et al., 2004; Lohnes, 2003; Subramanian et al., 1995; van den Akker et al., 2002). Deletions in the third cdx gene in mouse, Cdx4, do not cause homeotic transformations on its own, but its deletion can exacerbate the homeotic phenotype of cdx1 mutants (van Nes et al., 2006), suggesting redundancy in the activity of Cdx factors. In fact, embryos lacking the three cdx genes do not develop tissues past the otic vesicle, demonstrating Cdx redundant function in trunk and tail formation (Faas and Isaacs, 2009). Both tissue specification and patterning functions of Cdx are evolutionarily conserved, as similar phenotypes to those describe in mutant mice have been observed with other species lacking Cdx activities. In zebrafish (Davidson et al., 2003; Golling et al., 2002; Shimizu et al., 2005) as in Xenopus (Faas and Isaacs, 2009), loss of Cdx leads to posteriorized trunk and tail hox expression and a loss of posterior structures. For many hox genes, this regulation by Cdx is direct. In the mouse Cdx can directly bind to enhancers and regulate the expression of hoxa5, hoxb8,
and hoxc8 (Charite et al., 1998; Juan and Ruddle, 2003; Shashikant and Ruddle, 1996; Tabaries et al., 2005). It is unclear if Cdx also regulates other hox genes directly or not, as experiments to test direct binding and transcriptional activity of Cdx over other hox genes has not been carried out. Together these results suggest that Cdx family members play a role in patterning the A-P axis by regulating hox gene expression.

Given the important role of Cdx in regulating spinal cord specification and regionalization, considerable effort has been put into understanding cdx gene regulation. Transcription of cdx genes have been shown to be under the control of three signaling factors, RA, Wnt and FGF. RA regulation of Cdx genes is the most controversial, as current evidence support a role for RA in both activating and repressing cdx expression (Houle et al., 2000; Houle et al., 2003). In culture conditions, cells treated with RA activate cdx1 transcription (Houle et al., 2003), but in vivo, elimination of Retinoic Acid Binding Elements from the cdx1 promoter does not affect cdx1 expression (Houle et al., 2000). While it is possible that at different stages of development and in different tissues RA has positive or negative effects on cdx1 transcription, this has not been systematically tested. Thus, while RA is clearly a cdx gene regulator, systematic spatiotemporal analysis of RA function on cdx expression will be needed to fully understand RA’s role in cdx regulation.

Wnt signaling, on the other hand, has been shown to have clear activation functions on cdx. Loss of function experiments in mouse and zebrafish have shown that various Wnt ligands, particularly Wnt3a and Wnt8, are necessary for
cdx transcription (Pilon et al., 2006; Pilon et al., 2007; Shimizu et al., 2005). This activation has been shown to be direct, as several binding sites for Lef1/TCF3, the transcriptional regulators downstream of the Wnt signaling cascade, are present in cdx enhancer regions and are required for cdx transcription (Beland et al., 2004; Pilon et al., 2006; Pilon et al., 2007; Ro and Dawid, 2011). Together, this evidence supports a role for Wnts as cdx transcriptional activators.

FGFs have also been shown to be cdx transcriptional activators, although the molecular link between the FGF pathway and cdx activation is still missing. The first evidence for a link between FGF and cdx came from experiments in Xenopus. When Xenopus embryos were exposed to eFGF, the expression of Xcdx4 (previously known as Xcad3) and Xhox genes increased and expanded anteriorly (Pownall et al., 1996). Follow up experiments in chicken revealed similar activity for FGF: ectopic FGF2 and FGF4 in the nervous system rostrally expanded the expression domains of Cdx1, Cdx4 and Hoxb9 genes (Bel-Vialar et al., 2002). Importantly, the activation of Hox genes by FGF is Cdx-dependent, as FGF is unable to activate Hox gene transcription when Cdx activity is blocked (e.g., using a dominant-negative Cdx protein to interfere with endogenous Cdx function) (Isaacs et al., 1998). The inability of FGF to activate hox transcription in the absence of Cdx has led to the proposal of a linear relationship between FGF, Cdx and hox, in which Cdx mediates FGF patterning activity, and with the implication that once FGF activates cdx, FGF is no longer necessary for hox gene expression.
In this work I provide evidence to support Cdx4 role as a prime component of hindbrain and spinal cord patterning in zebrafish, working in conjunction with FGF signaling to regulate hox expression spatiotemporally. A comprehensive analysis of hox expression throughout early neural development in wild type and cdx4 mutant fish determined that three separate phases of hox expression were regulated by Cdx4 differentially. Cdx4 regulates the temporal induction and establishment of hox expression, and by doing so, regulates the spatial maintenance of hox expression. To further understand Cdx4 regulation of hox transcription, I analyzed the interactions between Cdx4 and FGF signaling in the control of hox spatiotemporal expression. cdx4 gain of function experiments allowed the ectopic activation of Cdx4 in the hindbrain, a tissue where it is not normally not present, as a proxy to study the interaction between Cdx4 and a molecular signaling cascade known to regulate posterior neural development; FGF. An analysis of hox expression, in the presence and absence of FGF signaling, supports a role of FGF in maintaining the Cdx4 positive feedback loop. Together the results place Cdx4 activity at a key developmental junction, partnering with FGF to regulate two processes: the specification of hindbrain and spinal cord territories, and subsequently, the several aspects of hox regulation essential for neural patterning.
**FIGURES**

**Figure 1.1:** Nieuwkoop’s activation-transformation model. Specification of the CNS is proposed to occur via two signaling steps. The first signaling step, known as “activation”, drives the initial induction of a neural population with anterior or forebrain characteristics. The second signaling step serves to establish more posterior regions and populations of the CNS by “transforming” or caudalizing the previously induced anterior neural tissue. Adapted from (Stern, 2001).

**Figure 1.2:** Cdx in the developing embryo. (A) Cdx transcription factors integrate molecular signals such as RA, FGF, and Wnt to regulate effector genes, known as hox genes, to coordinate key developmental processes. (B-I) Expression in red is of krox20, a marker for hindbrain rhombomeres 3 and 5. (B,D,F,H) Expression of cdx4 in the paraxial mesoderm and developing nervous
system of the zebrafish during early segmentation. (C,E,G,I) Expression of cdx1a in the paraxial mesoderm of the zebrafish during early segmentation.

Figure 1.3: The interaction between FGF, Cdx, and hox genes. (A) This linear model has been previously proposed as the method of interaction between FGF, Cdx, and hox genes. (B) This dissertation suggests that the interaction between FGF, Cdx, and hox is not a simple linear interaction, but includes multiple levels.
Chapter 2: Materials and Methods

Two different techniques were used to thoroughly assess Cdx4 regulation of hox genes in the zebrafish. First, a loss of function approach coupled with a comprehensive temporal analysis of hox gene expression was used to assay the regulatory role of Cdx4 on hox expression in the zebrafish. Hox gene expression was assayed at specific times during the hox expression window to determine spatial and temporal regulation by Cdx4.

In order to determine the extent to which Cdx requires FGF signaling to induce and maintain posterior hox genes gain and loss of function approaches were utilized to manipulate Cdx4 and FGF activity. Manipulations affecting FGF signaling and Cdx activity will be carried out at precise times during embryogenesis to avoid interfering with unrelated developmental processes and directly test FGF and Cdx function in hox transcriptional control.

Zebrafish lines

Zebrafish (Danio rerio) were raised and cared for following a standard protocol as previously described (Westerfield, 2000) following IACUC. Embryos for experiments were obtained by natural spawning, grown at 28°C, and staged as previously described (Kimmel et al., 1995). Three lines of fish were used in these studies. The wild-type line used was AB. Tg[hsp70:cdx4] (Fig. 2.1C), a line with a heat inducible cdx4 gene, was used as previously described (Skromne et al., 2007), and kugelig tv 205 (kggTv205, Fig. 2.1B), a Cdx4 mutant fish that only
expresses a truncated Cdx4 protein lacking the homeodomain, was used as well (Davidson et al., 2003).

**Gain of Cdx function - Transient cdx4 expression**

A standard heat-shock protocol was used to induce transient cdx4 expression (Fig. 2.1C), in which embryos were placed at an elevated temperature of 37°C for one hour (Halloran et al., 2000). Hours post-heat-shock (hphs) refers to the number of hours after transient expression of cdx4. Embryos were grown to 0, 1, 2, or 4 hphs, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for three days at 4°C, and then stored in Methanol until further processing.

**Loss of Cdx function - Cdx morpholino injections**

Cdx4 and cdx1a morpholino (Gene Tools LLC), a small antisense oligonucleotide that blocks cdx4 and cdx1a transcription respectively (Fig. 2.1A), was injected into one-cell stage wild-type and transgenic embryos. Embryos were injected with a final concentration of 4 ng/µl using a standard injection protocol (Bruce et al., 2001).
Loss of FGF function - SU5402 treatment

FGF pathway inhibition was achieved by treating wild-type and transgenic embryos with the chemical inhibitor SU5402, which interferes with the ATPase domain of FGF receptors (Mohammadi et al., 1997). The inhibitor was dissolved to a concentration of 20 mM in DMSO and stored at -80°C. SU5402 was applied to embryos at a concentration of 12.5 µM in embryo media + 0.1% DMSO at 6 hours post fertilization. Embryos were treated in the dark at 28°C until they were fixed in 4% paraformaldehyde at specific time points to be used in in situ hybridization.

Whole mount RNA in situ hybridization

cDNA probes were used to detect cdx4 (Joly et al., 1992); cdx1a (Shimizu et al., 2005); fgf8 (Reifers et al., 1998); fgf3 (Kiefer et al., 1996); hoxb and hoxc cluster genes (Prince et al., 1998a; Prince et al., 1998b); krox20 (Oxtoby and Jowett, 1993); myod; pea3 (Brown et al., 1998); in one and two color in situ hybridizations (ISH) as delineated in the figures and figure legends. ISH was conducted as previously described (Bruce et al., 2001) using NBT/BCIP or Fast Red.
Microscopy and photography

Embryos were deyolked using a small tungsten wire and flat mounted between coverslips. After preparation for imaging, embryos were photographed using a Zeiss Axiocam MRc camera on a Zeiss Discovery V20 microscope.
Figure 2.1: Gain and loss of function approach to Cdx studies. (A) A translation blocking cdx4 morpholino was used as a loss of function approach, in which the production of the endogenous Cdx4 protein was disrupted. (B) A mutant zebrafish, $k_{gg}^{10205}$, was also used as a loss of function approach, in which the mutation within the gene leads to the translation of a truncated Cdx4 protein that is unable to properly function in the absence of its’ homeodomain. (C) The gain of function approach for these studies was attained using a cdx4 transgene driven by a heat shock promoter that is activated by an elevated temperature. The translation blocking morpholino mentioned in (A) is unable to bind to the start site of translation in the transgenic cdx4, therefore it is ineffective in blocking the transgene.
Chapter 3: Cdx4 serves as a differential regulator of hox spatiotemporal expression.

Because Cdx4 regulates so many hox genes, Cdx4 can be used as a powerful tool to reveal general principles of hox cluster regulation and to distinguish modes of regulation both among and within clusters. Some principles may apply to all the genes in all the hox clusters. Some may apply to all the genes within one specific hox cluster. Some may apply to individual hox genes within one hox cluster, revealing gene-specific regulation. For instance, gene-specific regulation would be revealed if a gene or genes are not regulated concurrently with others in the same cluster. These principles are revealed by assessing the degree in which Cdx4 regulates hox temporal-spatial order and axial position of expression.

The accuracy of the tests critically depends on comparing wild type and cdx4 null mutant embryos that are precisely stage-matched. Precision is crucial because the spatiotemporal patterns of hox gene expression are highly dynamic. Small temporal differences in embryonic stage can result in large spatial differences in expression. To ensure accuracy, we took two precautionary measures. First, we analyzed only those wild type and mutant siblings that were obtained from cdx4 heterozygous parent crosses that were grown and processed together under identical conditions. Such embryos are not affected by an experimental manipulation such as injection of morpholinos that can delay development (personal observation), and they were genotyped at the conclusion of the experiment.
of the experiment. This measure assures synchronous development, so that consistent, differential expression in mutants can be ascribed directly to their genetics.

Second, we assess expression patterns of individual genes using previously established methods of classification. Three expression phases have been defined in mouse: induction, establishment and maintenance (Deschamps et al., 1999). In the induction phase, transcription of a particular hox gene is spatially restricted to neural and mesodermal precursors confined to the posterior primitive streak (gastrula margin in zebrafish) or the tailbud. This phase begins with the transcriptional activation and continued expression of a particular hox gene in the precursor cells only; the cellular descendants that will differentiate into spinal cord and mesoderm, lack expression. In the establishment phase these cellular descendants begin to transcribe the same hox genes that were expressed in their precursors. Descendants closest to the precursor cells start transcribing hox genes before distant descendants, so that the overall domain of expression gradually expands toward the anterior. The maintenance phase is defined as the time when the axial expression domain of a particular hox gene becomes stable. Thereafter, hox gene transcription stops and the expression domain disappear. Using this classification, we characterized the expression of each hox in zebrafish and compared these patterns to those in Cdx4-deficient embryos, to evaluate regulation of hox expression domains.
The three distinct phases of hox expression in fish

Although anterior hox gene expression patterns have been described in both mouse and zebrafish, central and posterior expression pattern are known only for mouse (Prince et al., 1998b). Our examination of hox patterns in zebrafish establishes that the normal spatiotemporal collinear order of hox expression is similar. Temporally, the expression patterns of central and posterior hox genes change dynamically, with key changes defining induction, establishment and maintenance phases, similar to those reported in mouse (Deschamps et al., 1999). Induction of central hox genes precedes the induction of posterior hox genes, causing their expression phases to be out of register. Initially during induction, hox gene expression is transient, appears simultaneously in neural and mesoderm precursor cells, in a rapid temporal sequence that matches the gene’s collinear order (hoxc4a, hoxc6a, hoxc8a, hoxc10a; Fig. 3.1). Induction of central hox genes takes place during mid-gastrulation, their expression confined to neural and mesoderm precursors: Cells that leave the precursor region in the gastrula margin down-regulate hox genes expression (Fig. 3.1). Induction of posterior hox genes takes place during early segmentation, their expression confined to neural and mesoderm precursors in the tailbud (Fig. 3.1J-L, 3.2C). During establishment, hox expression continues in precursor cells and new expression domains appear in maturing spinal cord and pre-somatic cells. This new expression expands from posterior to anterior regions during segmentation (Fig. 3.2). During maintenance, at late segmentation, hox genes are expressed in their definitive patterns in
spinal cord and somites, in a spatial order parallel to the gene’s collinear order (Fig. 3.2I-L, 3.3C-H). These results show three temporal processes establishing central and posterior hox genes spatial collinear expression. The identification of three similar expression phases of hox expression in zebrafish and in mouse suggests evolutionary conservation of the processes establishing central and posterior hox gene spatial collinear expression.

**Regulation of paralogous group expression and boundary position by Cdx4**

Our first studies focused on the anterior limit of expression attained at the maintenance phase, since this is the phase where the effect of Cdx4 has been most extensively analyzed in other organisms (Bel-Vialar et al., 2002; Faas and Isaacs, 2009; Gaunt et al., 2005; Isaacs et al., 1998; Young et al., 2009). In *Xenopus* and mouse, Cdx4 expression was eliminated, but expression of only a subset of genes was analyzed, and their expression was shifted toward the posterior (Bel-Vialar et al., 2002; Faas and Isaacs, 2009; Isaacs et al., 1998). The assumption has been that not only the genes studied, but all of the paralogous genes were shifted in concert. To test this assumption, we assessed the final anterior expression position of all forty-nine zebrafish hox genes. This analysis revealed a differential regulation across paralogs, but similar regulatory trends across all seven clusters (Fig. 3.3, 3.4). In each cluster, the anterior expression limit of hox paralogous groups 1 through 4 fails to shift (Fig. 3.3A, B, and data not shown). The central hox genes, comprising genes in paralogous
groups 5 through 10, were shifted posteriorly (Fig. 3.3C-F, 3.4, and data not shown). These genes correspond to those studied previously in other species (Bel-Vialar et al., 2002; Faas and Isaacs, 2009). The posterior hox genes, comprising genes in paralogous groups 11 through 13, while present in the tailbud were entirely absent from axial tissues (Fig. 3.3G, H and data not shown). These results establish a differential regulation of paralogous group genes, and establish that the regulation is similar from cluster to cluster.

Although the anterior expression boundary of anterior hox genes does not shift in the absence of Cdx4, a subset of these genes do develop a larger area of expression. Genes in paralogous group 4 consistently expand their expression toward the posterior (Fig. 3.3A, B, and data not shown). This finding pinpoints a group of genes that have their anterior border within the hindbrain, and are thus assumed to have a primary function in rhombomere patterning. In addition, this finding also shows that these genes are likely to be regulated by Cdx4 and play a role in patterning spinal cord.

As expected from prior studies of central genes belonging to paralogous groups 5 through 10, in the absence of Cdx4, the anterior boundary of expression was shifted toward the posterior. However, our detailed analysis identified consistent differences in the degree of shift among the genes in paralogous groups. The most distinctive difference correlated with the site of gene activity. Paralogous group 5 genes, in which the anterior expression border is in cervical regions, shifted only slightly at the limit of detection (one to two somite lengths, Fig. 3.3C, 3.4). In contrast, the remaining central hox genes
belonging to groups 6-10, in which the anterior expression boarder is in thoracic regions, shifted by 2-11 somite lengths (Fig. 3.3D-F, 3.4). Moreover, those genes with their anterior border in the thoracic region display an additional consistent pattern: the size of the shift progressively increases from anterior to posterior, so that the more 3’ genes that are expressed in the axis more anteriorly have less of a shift compared to more 5’ genes whose expression in the axis is more posterior (Fig. 3.3D-F, 3.4). These results suggest that, across each cluster, there is a gradient of differential sensitivity to Cdx4 regulation.

Loss of Cdx4 most severely altered expression of posterior hox genes belonging to paralogous groups 11-13, significantly reducing expression within the tailbud and completely abolishing axial expression. In contrast to the central genes that exhibited specific changes in expression across different paralogous groups, the posterior paralogous genes 11 to 13 were regulated in concert (Fig. 3.3G, H, and data not shown). They failed to display gene-specific differences in expression, suggesting that Cdx4 regulates the posterior hox gene expression similarly. The spatial expression patterns at this maintenance phase, in which expression is reduced in tailbud but is completely absent axially, suggests differential regulation. It implies that Cdx4 is dispensable for the expression of posterior hox genes in tailbud precursor cells, but is essential for the continual expression of these genes in differentiated cells. Moreover, these results are also in accord with the suggestion that, across each cluster, there is a gradient of differential sensitivity to Cdx4 regulation, with the more posterior genes having greater sensitivity. For posterior paralogous genes, the loss of Cdx4 more
severely diminishes expression, compared to the response of the anterior and central hox genes. These results thus further support a gradient of Cdx4 activity across hox clusters.

**Spatial collinear order of hox expression is not regulated by Cdx4**

In previous studies using only subsets of genes, the spatial order of hox collinear expression was considered to be independent of Cdx4 (Bel-Vialar et al., 2002; Faas and Isaacs, 2009). In those studies, the normal order of expression was seen, with 3' genes expressed anterior of 5' genes. However, the different effects that loss of Cdx4 had on the expression of anterior, central and posterior hox gene expression raises the possibility that Cdx4 could regulate spatial collinear order. If so, in Cdx4-deficient embryos, the spatial order of hox expression domains would be out of sequence. To test for altered collinear expression in Cdx4-deficient embryos, we analyzed the spatial organization of all expression domains within each cluster individually. This analysis established that, despite the different Cdx4-dependent alterations in hox gene expression, the spatial collinear order of gene expression in every cluster was preserved (Fig. 3.3A, C-E, 3.4). The preservation of the spatial order of hox expression in Cdx4-deficient embryos shows that during the maintenance phase, hox spatial collinear expression is not regulated by Cdx4.
Coordination of hox patterns across tissue is Cdx4-independent

In addition to the position and order of hox expression domains within neural and paraxial mesoderm tissues, accurate patterning of the embryo requires that hox expression domains are congruent and match between tissues. Congruency between spinal cord and somite domains could depend on the presence, in both tissues, of a common hox regulator. A putative common regulator, to coordinate hox expression domains in neural and pre-somitic mesoderm tissues, is Cdx4. Both tissues express cdx4 and both require Cdx4 for their patterning ((Gamer and Wright, 1993); Fig. 3.3C-H, 3.4). If Cdx4 functions to coordinate hox expression domains across tissues, then loss of cdx4 will change hox expression patterns in each tissue to a different extent causing mismatches in hox expression patterns across tissues. To assess if Cdx4 functions in coordinating hox patterning across tissues, we compared, in embryos lacking Cdx4, the changes in anterior limit of hox expression across neural and pre-somitic mesoderm tissues. In all but two cases the shift in the anterior limit of hox expression in both tissues matched (Fig. 3.3C-H, 3.4). The two exceptions were hoxc6b and hoxa9a, in which the anterior limit of hox expression shifted posteriorly in the spinal cord by 11 and 6 somite lengths respectively, but shifted posteriorly in the segmented paraxial mesoderm by mere 2-3 somite lengths (Fig. 3.3F, 3.4). For these two genes the shift in neural expression was significantly greater than the shifts in expression of other group 6 and 9 genes (2-3 somite lengths, Fig. 3.3E-F, 3.4). Notably, hoxc6b expression initially reached an anterior limit at somite level 5-6 similar to that of other
paralogous group 6 genes, but the anterior expression was prematurely lost suggesting some Cdx4 sensitivity to maintaining expression of this gene (Fig. 3.6D, H, L). The exceptional shift in \textit{hoxc6b} and \textit{hoxa9a} neural expression suggests a distinctive and unique role of Cdx4 in these genes’ neural regulation. The comparable shift in expression of most \textit{hox} genes in Cdx4-deficient embryos establishes that corresponding \textit{hox} expression pattern across spinal cord and somitic tissues are not regulated by Cdx4.

\textbf{Temporal activation of \textit{hox} expression in neural but not paraxial mesoderm tissue requires Cdx4}

The Cdx4-dependent changes in \textit{hox} expression patterns could have originated earlier in development, during the phases of induction or establishment. During induction, \textit{hox} transcription initiates in a temporal sequence of expression (Fig. 3.1). Because embryos develop in an anterior to posterior direction, time of induction is reflected in the anterior-to-posterior position of expression: The earlier in development axial progenitor cells initiate \textit{hox} gene transcription, the more anterior the \textit{hox} expression domain. Thus, delays in \textit{hox} gene induction in Cdx4-deficient embryos would directly translate into posterior shifts in \textit{hox} expression domains. If so, then the initiation of \textit{hox} expression in embryos lacking Cdx4 should be delayed. When all the central genes in the \textit{hoxba} and \textit{hoxca} clusters were examined, all of the genes were found to be delayed within one tissue only. Remarkably, delays were confined to the neural tissue; the temporal expression of these genes was normal in the pre-
somitic mesoderm (Fig. 3.5 and data not shown). This temporal delay in \textit{hox} transcription initiation can therefore account for the shift in spatial expression domain seen later in the spinal cord, implying that Cdx4 is important to regulate the timing of \textit{hox} transcriptional activation in the neural tissue.

During induction, the time of each gene’s expression matches each gene’s collinear order in the \textit{hox} cluster, but how that order is regulated is unknown. If the regulation is through Cdx4, then eliminating \textit{cdx4} should scramble the induction order or result in simultaneous induction. Neither result was found while examining induction of central \textit{hoxba} and \textit{hoxca} genes in the neural tissue, despite the fact that induction of these genes was delayed (Fig. 3.1, Fig. 3.2A-D, M-P). All the genes were expressed in their normal temporal order, collinearly. This preservation of \textit{hox} temporal collinear expression in Cdx4 deficient embryos indicates that in neural tissue, temporal collinear expression is not under Cdx4 control.

**Progressive establishment of \textit{hox} expression patterns requires Cdx4**

In the somitic mesoderm, Cdx4-dependent posterior shifts in \textit{hox} expression that are not explained by delays in \textit{hox} temporal induction might be explained by defects during the establishment phase, when the most anterior limit of \textit{hox} expression is reached through an anterior expansion of expression domains (Deschamps et al., 1999). If the onset or the rate of expansion were delayed, then the \textit{hox} expression domains would be stunted and shifted
posteriorly. Onset and rate could well be under regulation of Cdx4, since Cdx4 is expressed in pre-somatic mesoderm during the establishment phase (Gamer and Wright, 1993). In the mesoderm of Cdx4-deficient embryos, hox expression patterns were altered from the onset of the establishment phase, both temporally and spatially (Fig. 3.6). Temporally, hox expression domains failed to expand anteriorly during gastrulation and segmentation, resulting in reduced hox expression domains. The reduction persisted throughout the establishment phase. Spatially, the hox ladder-like expression in the anterior compartment of the most recently formed somites failed to resolve properly, so that each band remained indistinct (Fig. 3.6). These changes show that Cdx4 regulates hox establishment in the pre-somatic mesoderm. The lack of spatial resolution of hox expression in somites indicates a role for Cdx4 during somite patterning.

In neural tissue, the Cdx4-dependent delay in hox induction concordantly produced a delay in the establishment phase, which was expected, but also altered collinear expression, which as unexpected. Normally, during establishment of hox expression domains, the order in which each gene’s expression domain expands accords temporally with its collinear order within the cluster: Genes at the 3’-end expand their expression domain before genes at the 5’-end (Fig. 3.2). This temporal order was lost in Cdx4-deficient embryos. In the neural tissue, the expression domain was out of order for at least two genes within the hoxca cluster (hoxc6a and hoxc8a; Fig. 3.6). In this cluster, the 5’-end gene hoxc8a expanded before its adjacent 3’-end gene hoxc6a (Fig. 3.6A, B, E, F, I, J). Remarkably, these alterations in the collinear expansion failed to affect
the later spatial collinear expression; in all cases, the final collinear pattern was corrected to reflect normal spatial expression (Fig. 3.6E, F, I, J). These changes show that Cdx4 is required in neural tissue for the normal, orderly progression of *hox* gene expression.
Figure 3.1: Induction of hox expression in the zebrafish. (A,D,G,J) Early, (B,E,H,K) middle, and (C,F,I,L) late gastrula stage embryos showing the expression of the stated hox gene in black and myoD(red) to label the somites. The temporal activation of hox genes in the zebrafish correlate with the location of the gene on the cluster. More 3’ genes, like hoxc4a, are turned on earlier (A), whereas more 5’ genes, such as hoxc10a, are turned on later as evident by the lack of expression even at the end of gastrulation (L). Scale bar represents 0.2 mm
Figure 3.2: Establishment of hox expression in the zebrafish. (A-D) Early, (E-H), middle, and (I-L) late segmentation stages of zebrafish development showing the expression of the various labeled hox genes in purple and krox20 (red) to label rhombomeres 3 and 5 of the hindbrain. The expansion of hox expression to its’ anterior boundary of expression occurs during segmentation and is hallmarked by the spread of more posterior genes before more anterior genes. Scale bar represents 0.2 mm.
Figure 3.3: Differential regulation of hox maintenance by Cdx4. (A-H) Late segmentation zebrafish embryos showing the comparison between normal hox expression (wt) and mutant embryos lacking Cdx4 (kgg). Cdx4 has very little affect on the expression of anterior hox genes (A,B) in that the only changes, if any, were on the posterior limit of expression (B). On middle hox genes (C-F) the lack of Cdx4 led to a posteriorized level of expression in the developing CNS and paraxial mesoderm. Posterior hox genes (G-H) were never expressed in differentiated tissue in Cdx4 mutant embryos. Scale bar represents 0.2 mm.
Figure 3.4: Global regulation of hox expression by Cdx4. Graph representing the neural and mesodermal spatial expression domain (x-axis, in somite units) of central hox genes (y-axis) in wild type and Cdx4-deficient (kgg^{hi216A} mutant) embryos. Anterior is to the right. For each gene, spinal cord is represented as a large rectangle, and the somites and unsegmented paraxial mesoderm as small rectangles. Black region represents the change in expression pattern between wild type and Cdx4-deficient embryos. Gray region represent the region of
expression in both conditions. Bold lines distinguish each gene cluster. Each point is the average of 20 embryos, rounded to the closest integer. Differences observed in hox gene expression patterns between wild type and Cdx4-deficient embryos were all significant based on Fischer’s exact test (neural tissue, $p \leq 0.00067$; paraxial mesoderm, $p \leq 0.00013$)

**Figure 3.5:** Cdx4 regulates the temporal induction of hox expression in the zebrafish. (A,D,G) Early, (B,E,H) middle, and (C,F,I) late gastrula stage embryos showing the expression of the stated hox gene in black and myoD(red) to label the somites. The temporal activation of hox genes in the zebrafish are delayed in the absence of Cdx4. Hoxc4a, hoxc6a, and hoxc8a, which were all shown to be induced at early gastrulation in the wt, are delayed and are not activated until mid(B,E) or late (I) gastrulation. Furthermore, by the end of gastrulation none of these gene have been induced in the putative developing nervous system. Scale bar represents 0.2 mm.
Figure 3.6: Cdx4 regulates the time of hox establishment. (A-D) Early, (E-H), middle, and (I-L) late segmentation stages of zebrafish development in Cdx4 mutants showing the expression of the various labeled hox genes in purple and krox20 (red) to label rhombomeres 3 and 5 of the hindbrain. The expansion of hox expression to its’ anterior boundary of expression is delayed in comparison to wt. Scale bar represents 0.2 mm.
Chapter 4: Cdx and FGF interactions establish a molecular switch for posterior nervous system specification

Our results in zebrafish (Chapter 3), as well as other studies in mouse, *Xenopus*, and (Bel-Vialar et al., 2002; Charite et al., 1998; Gaunt et al., 2008; Isaacs et al., 1998; Pownall et al., 1996), show that Cdx transcription factors are essential part of the mechanisms regulating accurate *hox* gene expression. However, how these mechanisms operate, and what is the role of Cdx in these processes remains unknown. Furthermore, given the essential role of Cdx in *hox* transcription, it is key to understand how *cdx* expression is regulated. Several studies in Xenopus and chicken, attempting to address these questions, have given us a general understanding of *hox* gene regulation by Cdx (Bel-Vialar et al., 2002; Isaacs et al., 1998; Pownall et al., 1996). However, each of these studies utilized chimeric Cdx proteins that do not reflect the true function of the endogenous protein. To assess the role of native Cdx4 on the regulation of *hox* genes, we utilized a transgenic version of *cdx4* to gain spatial and temporal control of *cdx4* activity. These approaches provide new avenues to test Cdx4 activity in natural and ectopic locations at different stages of development.

To dissect the spatiotemporal regulation of *hox* genes in the CNS, we compared *hox* expression dynamics after ectopic overexpression of the only *cdx* gene transcribed in zebrafish neural tissue, *cdx4* (Skromne et al., 2007). *Cdx4* was ectopically expressed for 1-hour at the end of gastrulation (tailbud stage, 10 hours post fertilization, hpf) using a heat-inducible transgene, a treatment shown
to posteriorize the hindbrain (Skromne et al., 2007). Embryos were then analyzed at 0, 1, 2 and 4 hours post heat shock (equivalent to 3-, 6-, 8- and 12-somite stages) for the expression of known Cdx4 target genes, hoxb6a, hoxb8a, hoxb9a and hoxb10a (Davidson et al., 2003). As a control, we also monitored the expression of an anterior hox gene whose regulation is independent of Cdx4, hoxb1a (Moens and Prince, 2002). The expression of hoxb1a was not affected by cdx4 transgene induction (Fig. 4.1 A, G, I, M, Q). Temporally, posterior hox genes were expressed sequentially. Immediately after induction we detected hoxb6a and hoxb8a expression, followed by hoxb9a and hoxb10a expression 2-hours later (Fig. 4.1; hoxb6a 0hphs n=23/59; hoxb8a 0hphs n=32/65; hoxb9a 2hphs n=32/62; hoxb10a 2hphs n=25/58). Spatially, immediately after induction, hoxb6a and hoxb8a expression was ubiquitous, but within 2 hours their expression became restricted specifically to the telencephalon’s anterior neural ridge, the midbrain/hindbrain boundary and hindbrain’s rhombomere 4 (Fig. 4.1 J, K, N, O). In comparison, hoxb9a and hoxb10a expression were never ubiquitous, rather their expression was restricted to the same areas as hoxb6a and hoxb8a from the time of detection at 2 hours post induction (Fig. 4.1 L, P). The hox temporal sequence of activation followed the order of genes in the cluster, from 3’ to 5’ end, suggesting temporal regulation of ectopic hox gene expression. The similarities in hox expression after cdx4 induction and during normal development suggest conservation of hox regulatory mechanisms and
further justify the use of our method of \textit{cdx4} overexpression as a tool to understand Cdx and \textit{hox} gene regulation.

\textbf{Cdx positive feedback loop required to maintain \textit{hox} expression}

The late restriction in \textit{hox} expression is inconsistent with the initial ubiquitous activation of the \textit{cdx4} transgene, implicating additional regulatory mechanism. One possible mechanism is \textit{cdx} positive feedback regulation required for their continuous expression during posterior embryo development (Beland et al., 2004; Prinos et al., 2001). To investigate \textit{cdx} positive feedback regulation during the restriction of \textit{hox} expression domains, we analyzed the expression of the two \textit{cdx} genes involved in spinal cord specification and patterning, \textit{cdx1a} and \textit{cdx4} (Skromne et al., 2007). While \textit{cdx1a} was not induced by the \textit{cdx4} transgene (Fig. 1.2), \textit{cdx4} transcripts were dynamic (Fig.4.2B, D). Initially, \textit{cdx4} expression was ubiquitous (Fig. 4.2B), but by 4 hours after transgene induction the expression was restricted to the same domains of ectopic \textit{hox} expression (Fig. 4.2D). To investigate whether \textit{cdx4} transcripts originated from the endogenous gene or the transgene, we repeated the experiment in embryos lacking endogenous Cdx4 activity with the expectation that any expression depending on the endogenous gene would be lost. Transgene induction in embryos lacking endogenous Cdx4 resulted in ubiquitous \textit{cdx4} expression that faded and was not maintained in restricted expression domains (Fig. 4.3B, D). These changes in \textit{cdx4} show that \textit{cdx} positive feedback regulation does not involve Cdx1a, but require endogenous Cdx4. Furthermore, endogenous \textit{cdx4} is also required for maintenance of \textit{hox} expression.
Cdx positive feedback regulation requires active FGF signaling

Cdx4 positive feedback regulation explains the persistence of cdx4 and hox transcripts many hours after transgene induction, but does not explain their restricted expression to the anterior neural ridge, midbrain/hindbrain boundary, and rhombomere 4. To begin investigating the mechanisms of spatial regulation, we took a candidate gene approach to identify genes whose expression was shared among all these regions. The only set of genes meeting these criteria belonged to the FGF pathway and included FGF3, FGF8a (Kudoh et al., 2002; Reifers et al., 1998); Fig. 4.4), FGF Receptors-1 and -2 (FGFR; (Ota et al., 2010; Tonou-Fujimori et al., 2002) and its downstream effectors erm, pea3 (Roehl and Nusslein-Volhard, 2001); Fig. 4.4) and spry1 (Komisarczuk et al., 2008). The colocalization of ectopic cdx4 and hox expression to areas of FGF activity, together with previous published data showing that in the tailbud FGF regulates cdx expression (Shimizu et al., 2006), suggests that the spatial regulation of ectopic cdx4 and hox may be under FGF control. To functionally test whether FGF activity is necessary for cdx4 and hox restricted expression, we induced transgene expression in the presence of an FGF-pathway inhibitor and monitored changes in the spatial distribution of cdx4 and hox transcripts. If FGF activity is necessary for cdx4 and hox restricted expression, their expression would be lost in the presence of the inhibitor. To block FGF pathway activity embryos carrying the cdx4 transgene were treated at the beginning of gastrulation (6 hpf) with the FGF-receptor inhibitor SU5402 (Mohammadi et al., 1997). Then, cdx4 was
overexpressed by inducing the transgene at the end of gastrulation for 1-hour (10-11 hpf). Expression of cdx4, hox, and the FGF target gene pea3 were analyzed 1-, 2- and 4-hours after induction (Fig. 4.5 and data not shown). This regiment was effective in blocking FGF pathway activity, as shown by the continual expression of fgf3 in neural tissue but the complete loss of neural pea3 expression at the onset of segmentation, the time at which the cdx4 transgene was induced (data not shown). Similar to cdx4 overexpression in the presence of an intact FGF pathway, in the presence of the FGF pathway inhibitor, the cdx4 transgene induced cdx4 and hoxb6a expression ubiquitously (Fig. 4.5F and data not shown). However, by 2 hours post induction embryos lacking FGF activity did not express cdx4, hoxb6a, hoxb9a or hoxb10a ectopically in restricted domains (Fig. 4.5N-P, R-T). The early ubiquitous activation of cdx4 and hox expression in the absence of FGF activity suggests that FGF signaling is not necessary for hox gene induction by Cdx4. The loss of the late restricted expression suggests that FGF is necessary for maintenance of cdx4 and hox spatial expression domains by maintaining the Cdx4 positive feedback loop.

**FGF and Cdx must cooperate to maintain hox expression**

Our gain and loss of function results support a model in which Cdx4 is necessary for temporal induction and spatial maintenance of hox expression, and that FGF activity is only required for the spatial maintenance of hox expression by helping sustain Cdx4 positive feedback. However, it remains unclear whether
these interactions discerned in an ectopic location are functioning in the native tissues where spinal cord is generated and patterned, the caudal neural plate and tailbud. Based on our model, we predicted that in the caudal neural plate and tailbud, FGF pathway inhibition would not affect \textit{cdx4} and \textit{hox} genes’ temporal induction but eliminate their spatial maintenance.
**Figure 4.1:** Induction of middle *hox* genes by *cdx4* is temporally sequential, but their maintenance is spatially restricted to specific regions. Spatiotemporal analysis of *hoxb1a*, *hoxb6a*, *hoxb9a* and *hoxb10a* expression in embryos that have been induced to over-express *cdx4* and were subsequently analyzed immediately (E-H), one hour after (I-L), two hours after (M-P), and four hours after(Q-T) *cdx4* over-expression. (A-D) transgenic embryos at tb stage showing
expression of hox genes before cdx4 over-expression (black box). (E, I, M, Q, U) Ectopic expression of cdx4 does not induce hoxb1a expression in ectopic locations. (F,J,N,R) Cdx4 induces hoxb6a expression broadly throughout the nervous system immediately after over-expression, but within one hour after induction the expression of hoxb6a begins to fade to more specific regions. (G,K,O,S) hoxb9a induction by Cdx4 does not occur until two hours after over-expression and is induced in specific regions and subsequently maintained in these same areas. (H,L,P,T) hoxb10a induction by Cdx4 does not occur until two hours after over-expression and is induced in specific regions and subsequently maintained in these same areas. (U-X) Wild-type expression of posterior hox genes in embryos staged (15hpf) to match those of embryos at 4 hours after cdx4 over-expression show no expression of these genes (black box) Embryos costained with krx20 (red) to show rhombomeres 3 and 5 in hindbrain. Arrows indicate induction of posterior hox gene. Asterisks indicate areas of posterior hox gene maintenance. Embryos are flat mounted with anterior to the left. Scale bar represents 0.2 mm. hoxb6a 2hphs n= 29/60 hoxb8a 2hphs n= 29/60 hoxb9a 2hphs n=31/60

**Figure 4.2:** Cdx4 expression is maintained in specific regions after over-expression. The expression of cdx4 at early (A-B) and mid-(C-D) segmentation. Immediately after over-expression cdx4 is expressed uniformly throughout the CNS (B) as compared to the wild-type expression in the posterior of the embryo (A), but this expression becomes restricted to very specific regions of the CNS by mid-segmentation (D). These regions include the telencephalon (T), midbrain/hindbrain boundary (M/H), rhombomere 4 of the hindbrain (r4), and the spinal cord. Expression is red is of krox20 to label rhombomeres 3 and 5.
Figure 4.3: Ectopic Cdx4 expression is lost in the absence of the Cdx4 feedback loop. The expression of cdx4 at early (A-B) and mid-(C-D) segmentation. Immediately after over-expression cdx4 is expressed uniformly throughout the CNS (A). After over-expression in the absence of endogenous Cdx4, the expression is still uniform throughout the CNS (B). However, in the absence of the expression of the endogenous cdx4 gene, the expression of cdx4 is not retained in the specific anterior regions of the CNS (D) as seen in the over-expression experiments where the endogenous gene expression is uninterrupted (C). Expression is red is of krox20 to label rhombomeres 3 and 5.
**Figure 4.4:** Regions of Ectopic cdx4 expression correlate with areas of FGF activity. The expression of cdx4, FGF3, FGF8, and pea3 at mid-segmentation. Expression in red is of krox20 to label rhombomeres 3 and 5.
Figure 4.5: FGF signaling is necessary for maintenance of middle hox gene expression. Spatiotemporal analysis of hoxb1a (A, E, I, M, Q, U), hoxb6a, hoxb9a and hoxb10a expression in embryos where FGF signaling has been blocked (SU5402) and subsequently induced to over-express cdx4 were analyzed immediately (E-H), one hour after (I-L), two hours after (M-P), and four hours after(Q-T) cdx4 over-expression. (A-D) Expression of hox genes in transgenic embryos treated with SU5402 at tb stage, but without over-expression of cdx4. (E, I, M, Q, U) Ectopic expression of cdx4 in the absence of FGF signaling does not induce hoxb1a expression in ectopic locations. (F,J,N,R)
Cdx4 induces *hoxb6a* expression broadly throughout the nervous system immediately after over-expression, but within one hour after induction the expression of *hoxb6a* begins to fade and is not maintained in any ectopic regions. (G,K,O,S) *hoxb9a* induction by Cdx4 does not occur in any ectopic locations. (H,L,P,T) *hoxb10a* induction by Cdx4 does not occur in any ectopic locations and is therefore not maintained either. (U-X) Wild-type expression of posterior *hox* genes in embryos staged (15hpf) to match those of embryos at 4 hours after cdx4 over-expression show no expression of these genes (black box). Embryos costained with *krox20* (red) to show rhombomeres 3 and 5 in hindbrain. Arrows indicate induction of posterior *hox* gene. Embryos are flat mounted with anterior to the left. Scale bar represents 0.2 mm.
Chapter 5: Discussion

In A-P development of the CNS, Cdx functions as a global regulator of hox genes and does so synergistically with FGF to regulate posterior neural identity. This dissertation utilizes two unique studies to analyze hox gene and Cdx4 regulation in the developing zebrafish nervous system. First, a comprehensive analysis of all seven hox clusters throughout the entire time of hox gene expression in the zebrafish provided key insight into the regulation of hox genes by Cdx4 along the A-P axis of the zebrafish. Second, FGF and Cdx4 interactions were analyzed in a unique study where the hindbrain was used as a proxy to understand and establish the relationship between FGF and Cdx outside of additional signaling contexts that may exist in their native region of interaction. Our results suggest that the conserved role of Cdx in neural development involves the family of transcription factors working to globally regulate hox gene expression and do so by integrating the key role of FGF in maintaining Cdx activity.

Evolutionary conservation of three phases of hox expression

Systematic spatiotemporal analysis of hox cluster transcription in zebrafish identified three phases of hox gene expression similar to those reported in mammals suggesting evolutionary conservation in the mechanisms regulating the spatial and temporal transcription of hox genes across vertebrates. Studies
in the mouse, chick, and *Xenopus* suggest that the dynamic nature of *hox* gene expression is dependent on the position of *hox* genes within the cluster (Duboule and Dolle, 1989), and is regulated in three separated phases: induction, establishment, and maintenance (Bel-Vialar et al., 2002; Deschamps et al., 1999; Gaunt and Strachan, 1994, 1996; Pownall et al., 1998). Induction, which is defined as the activation of the gene and is hallmarked by a temporal onset of transcription, that correlates with the location of the gene on the cluster: genes at the 3' end of the cluster become transcriptionally active earlier in development than genes at the cluster's 5' end (Gaunt and Strachan, 1996; van der Hoeven et al., 1996). The second phase, establishment, is defined as the posterior-to-anterior expansion in *hox* expression domains (Bel-Vialar et al., 2002; Deschamps and Wijgerde, 1993; Gaunt and Strachan, 1994). This expansion is not the result of posterior cells moving to more anterior regions, but the result of a “traveling wave” of *hox* gene activation, from posterior to anterior (Deschamps and Wijgerde, 1993). The final phase, maintenance, fine-tunes *hox* transcriptional domains to their final definitive spatial pattern of expression (Bel-Vialar et al., 2002; Gaunt and Strachan, 1994). Here I identified, in a non-tetrapod, equivalent phases of *hox* gene expression. In zebrafish, despite the existence of additional *hox* clusters compared to tetrapods, *hox* genes are induced in a temporal sequence that corresponds to their cluster location, then, their expression domain expands anteriorly where it is maintained at particular axial positions.
Temporal collinearity of zebrafish hox induction

Zebralish hox genes are activated sequentially with a collinear correlation to the location of the gene on the cluster. Temporal collinear hox activation is a highly conserved method of hox induction found throughout vertebrates (Duboule, 1992; Duboule and Dolle, 1989; Gaunt and Strachan, 1996; van der Hoeven et al., 1996). Zebralish hox gene transcription is activated before gastrulation is completed (Fig. 3.1). The first genes activated are more 3’ genes of each cluster, which are turned on around mid-gastrulation. The more 5’ the gene along the cluster the later the expression during gastrulation with the most 5’ genes being activated shortly after gastrulation completes (Fig. 3.1, unpublished data). My comprehensive analysis of zebralish hox induction, taken into account of a very similar mechanism of hox activation throughout vertebrates, suggests that the mechanism(s) regulating hox gene induction are highly conserved.

Establishment of hox expression in the zebralish

The posterior to anterior expansion of hox gene expression in the zebralish characterizes an uncommon separation of temporal and spatial collinearity. After the temporal activation of more anterior genes before more posterior genes, it was assumed that due to the final nested spatial expression of zebralish hox genes, which follows spatial collinearity (Fig. 3.3; unpublished data), that the expansion of hox gene expression in the zebralish would follow a
similar collinear pattern. The coupling of temporal activation and spatial collinearity has been previously suggested in chick studies in which the transcriptional activation wave of 3’ genes begins before and progress more anteriorly than 5’ genes (Gaunt and Strachan, 1994, 1996). However, in the zebrafish the first hox genes to begin their anterior expansion are posterior or more 5’ genes (Fig. 3.4). This suggests an uncoupling of temporal and spatial collinearity and that the eventual spatial collinear pattern of hox genes along the A-P axis of the zebrafish embryo is regulated independently of the temporal activation of the same genes. The mechanistic separation of temporal and spatial collinearity has also been recently suggested in mice (Tschopp et al., 2009).

**Spatial collinearity is essential for hox gene maintenance**

The final spatial expression of zebrafish hox genes along the A-P axis are nested in a pattern that strongly correlates with the location of the gene along the cluster. More 3’ genes of the hox cluster are expressed and maintained in more anterior domains along the A-P axis versus more 5’ genes being expressed more posteriorly and nested within the more anteriorly expressed genes (Fig. 3.3 and data not shown). Hox genes, like their insect homologs, have been found to be spatially nested along the A-P axis throughout vertebrates (Duboule and Dolle, 1989). The concept in which 3’ genes are expressed more anteriorly and 5’ genes are expressed more posterior nested within the more anteriorly expressed
genes is known as spatial collinearity and is known to be regulated by the same two classes of genes in the insect and mammal (Deschamps et al., 1999). Similarity in the conserved spatial collinearity of hox gene expression between the zebrafish, mammals, and insects suggests that the mechanism(s) essential to establishing hox spatial expression are similar or conserved throughout the evolutionary process.

**Cdx: Global Regulator of hox genes**

Transcriptional control of hox genes by Cdx factors has been extrapolated to encompass all hox genes from the thorough expression analysis of only a handful of trunk and tail hox genes (Chawengsaksophak et al., 2004; Copf et al., 2004; Faas and Isaacs, 2009; Isaacs et al., 1998; Savory et al., 2009; Shimizu et al., 2006; Shinmyo et al., 2005; van den Akker et al., 2002; Young et al., 2009). Here, by carrying out a comprehensive analysis of expression of all hox genes in a Cdx4-deficient zebrafish background we have been able to identify global regulatory events affecting whole hox cluster transcription and distinguish them from local regulatory events affecting individual genes. In general, hox gene response to the loss of Cdx4 allows the grouping of hox genes into three large groups: hox 1-4 genes that have anterior limit of expression in the hindbrain, are for the most part not affected by the loss of Cdx4; hox 5-10 genes, whose anterior limit of expression locates in the trunk, are mildly affected by the loss of Cdx4 with their expression domains shifted posteriorly by no more than a handful
of somites; and hox 11-13 normally expressed in tail tissue, are never expressed in differentiated tissue in Cdx4-deficient embryos. Within these groups we were able to identify genes that are more sensitive to the loss of Cdx4 and therefore, behave in a different way than the majority of hox genes. Together these findings show that Cdx4 is a key global regulator of zebrafish hox gene transcription.

**Cdx4 regulation of hox 1-4 genes**

Cdx4 regulation of hox paralogs 1-4 is restricted to the posterior domain of expression of those hox genes, whose transcriptional expression domain extends from the hindbrain into the spinal cord. These genes include hox groups 3 and 4 and excludes hox gene groups 1 and 2 (Fig. 3.3A-B; 4.1A,F,K,P,U,Z and data not shown). This is consistent with the restriction of hox gene groups 1 and 2 expression to the hindbrain, outside of the cdx4 spinal cord expression domain. For hox groups 3 and 4 genes, Cdx4 regulates the posterior expression boundary. In the absence of cdx4, the posterior limit of hox genes 3 and 4 expands posteriorly, increasing their spatial expression domain.

Cdx4 could be regulating the expression of hox group 3 and 4 genes directly or indirectly. In the mouse Cdx can directly regulate the expression of hoxa5, hoxb8, and hoxc8 by binding to regulatory elements of these prospective genes (Charite et al., 1998; Juan and Ruddle, 2003; Shashikant and Ruddle, 1996; Tabaries et al., 2005). However, no Cdx transcription factor has been
found to directly regulate hox 3 and 4 genes. While it is possible that Cdx4 directly regulate expression of hox 3 and 4 genes through, as yet unidentified binding sites, a more plausible explanation is that Cdx4 regulates the posterior limit of hox 3 and 4 expression indirectly, through the regulation of posterior hox genes. Hox transcription factors can repress the expression of other hox genes. A well characterized example in the nervous system is the cross-repressive activity of Hoxc6 and Hoxc9 on each other in developing spinal motor neurons (Dasen et al., 2003).

**Cdx4 regulation of hox 5-10 genes**

Cdx4 regulates two phases of paralogous hox 5-10 gene transcription, the temporal induction and the anterior spreading of expression, in a tissue specific manner. In neural tissue, where cdx4 is the only Cdx family member transcribed (Fig. 1.2B,D,F,H), loss of Cdx4 causes temporal delays in the induction and spatial shifts in expression of hox 5-10 genes. In contrast, in the paraxial mesoderm, where both cdx4 and cdx1a are transcribed, loss of Cdx4 only results in delays of the anterior spread of hox expression domain during the establishment phase. These differences can be attributed to Cdx1a and Cdx4 redundancy, a phenomenon also observed in mouse paraxial mesoderm (van den Akker et al., 2002). Importantly, loss of Cdx1a neither affects the initiation or establishment of hox gene expression in paraxial mesoderm (Skromne et al.,
indicating that, for hox gene transcriptional initiation, Cdx4 and Cdx1a functions redundantly, but for the establishment only Cdx4 is required.

It is not likely that Cdx4 plays a direct role in regulating hox gene maintenance as hox genes are still maintained in a spatial collinear manner in the absence of Cdx4 (Fig. 3.3 & 3.4). This lack of hox maintenance regulation has been previously described in other zebrafish and mice studies (Skromne et al., 2007; van Nes et al., 2006). Furthermore, despite the differential regulation of hox genes by Cdx family members in neural and paraxial mesoderm tissues, the final hox expression domain continue to be aligned across tissues despite being shifted posteriorly (Fig. 3.4). These results do not show a strong correlation between hox maintenance in neural and paraxial mesoderm tissue and the presence of Cdx4 suggesting that the posteriorized expression domains of hox genes during the maintenance phase are an indirect result of Cdx4 regulation of hox 5-10 induction and establishment.

Cdx4 could be regulating hox 5-10 gene induction and establishment via direct and/or indirect mechanisms. As previously discussed, Cdx4 could directly regulate hox expression by regulating transcription (Charite et al., 1998; Juan and Ruddle, 2003; Schyr et al., 2012; Tabaries et al., 2005). This mechanism is further supported by overexpression studies I conducted, which show that Cdx4 overexpression directly correlates to trunk hox gene overexpression (Fig. 4.1 & 4.2). The opposing mechanism suggests that Cdx4 indirectly regulates trunk hox genes by directly regulating chromatin configuration. In a study of hox regulation in mouse spinal motor neurons, it was
determined that Cdx2 played an essential role in preparing hox chromatin for transcription by derepressing, through demethylation, and relaxation (Mazzoni et al., 2013). This mechanism could also fit the results I found for the trunk hox genes. In the absence of Cdx4 the hox chromatin, in neural precursors of the zebrafish, could remain tightly wound and repressed by methylation making it more difficult for the transcriptional machinery to bind and begin the transcription process. The machinery is not blocked, but delayed causing a delay in the activation, or initial transcription of hox 5-10 genes. As the embryo continues to develop the entire regulation of trunk hox genes is now delayed, which inadvertently leads to the posteriorization of the hox spatial domains. Although the direct and indirect mechanisms are separate it does not mean that Cdx4 functions one way or the other. It is possible that Cdx4 is concerting both mechanisms in its regulation of hox 5-10 genes. In the chromatin modification study conducted in mouse spinal neurons they also found that Cdx2 had multiple binding sites within the hox chromatin, which further supports the idea of concerted mechanisms (Mazzoni et al., 2013).

**Cdx4 regulation of hox 11-13 genes**

Cdx4 is critical for all phases of hox 11-13 gene expression, including the maintenance phase of transcription in differentiated tissues. This situation is unlike what is seen for hox 1-10 genes, making hox 11-13 genes the most sensitive genes to the loss of Cdx4. In Cdx4 deficient embryos, temporal
induction of \textit{hox} 11-13 genes are extensively delayed in neural tissue and are never expressed in tissues outside of the undifferentiated stem cells of the tailbud (Fig. 3.3 G-H and data not shown). This strong dependence of \textit{hox} 11-13 genes on Cdx4 could be attributed to two non-mutually exclusive regulatory processes. Transcription of \textit{hox} 11-13 genes could be under regulation of a limited number of transcription factors, of which Cdx4 is a primary regulator. In this way, Cdx4 elimination results in an almost complete abrogation of \textit{hox} 11-13 expression. Transcription of \textit{hox} 11-13 genes could also depend on the chromatin state of the \textit{hox} cluster, and the sequential 3'-to-5' activation of \textit{hox} genes (Mazzoni et al., 2013). In this scenario, delays in the activation of 3’ genes would result in an increased delay in the activation of 5’ genes, a snowball effect whose compounding effect would be manifest with the greatest severity in the most 5’, \textit{hox} 11-13 genes. Evidence in mouse provides support for both, direct transcriptional and indirect chromatin regulation of \textit{hox} genes by Cdx (Mazzoni et al., 2013).

\textbf{FGF and Cdx interactions regulate \textit{hox} gene induction and maintenance}

To further understand the regulation of \textit{hox} gene transcription by Cdx4, I used the developing hindbrain to study the interactions between Cdx4 and another \textit{hox} gene regulator, the signaling factor FGF, revealing that complex interactions between Cdx4 and FGF establish a regulatory network that specifies the correct time and place of \textit{hox} gene expression. Interactions between FGF
signaling and Cdx have been shown to be essential for the proper regulation of hox gene expression in Xenopus and chick (Bel-Vialar et al., 2002; Isaacs et al., 1998; Pownall et al., 1996). Based on these published results, it was proposed that Cdx functions downstream, and independently of FGF, to activate posterior hox genes, and that FGF’s only role is to induce Cdx. Here, by using the developing hindbrain as a proxy to study the interactions of FGF and Cdx4, a tissue that varies little in size and signaling centers compared to the spinal cord, I suggest that the interaction between FGF, Cdx, and hox genes is not linear. Instead, my results suggest that Cdx does not function independently of FGF as previously suggested, but requires FGF continuously for transcription and protein activity. Based on my results, I propose that Cdx4 actively and continuously requires FGF for the proper and sustained spatial expression of hox genes.

Cdx induction of hox genes is FGF independent

Cdx4 is required for trunk hox gene expression and my results indicate that, contrary to current models, this regulation is irrespective of FGF signaling status. It is well established that blocking FGF signaling results in a complete loss of posterior structures (Amaya et al., 1991; Griffin et al., 1995) and that these effects are mediated through the regulation of hox genes in the pre-somatic mesoderm and CNS (Bel-Vialar et al., 2002; Dubrulle et al., 2001; Kengaku and Okamoto, 1995; Lamb and Harland, 1995; Pownall et al., 1996). I find that in the absence of FGF activity the anterior limit of cdx4 and trunk hoxb gene expression
are posteriorized, but never lost (data not shown). Furthermore, when cdx4 is overexpressed the ectopic expression of the *hoxb* trunk genes correlates strongly to the expression of cdx4 in the presence or absence of FGF (Fig. 4.1 & 4.5F). These results do not rule out a role of FGF in regulating *cdx* and *hox* gene induction, but suggest that FGF is not required for the induction of either. Additionally, ectopic *hox* expression driven by *cdx4* overexpression follows a temporal collinear fashion in which more 3' genes of the cluster are expressed earlier and more broadly than 5' genes. A possible explanation for this differential ectopic induction could be the regulation of *hox* chromatin configuration and access by Cdx. These results further support a direct and indirect role of Cdx4 in regulating *hox* genes, but more importantly, FGF activity is not an essential component to the role of Cdx4 in *hox* gene induction of the zebrafish.

**Maintenance of hox expression requires Cdx and FGF activity**

FGF activity is required to maintain *cdx4* and trunk *hox* gene transcription in a positive autoregulatory loop that requires Cdx4 protein activity. Cdx family members have been shown to utilize autoregulatory mechanisms to maintain their expression (Prinos et al., 2001; Xu et al., 1999). I find that, in Cdx4 deficient embryos, transcription of the *cdx4* gene prematurely stops at the end of gastrulation. Furthermore, when transgenic *cdx4* is overexpressed in the absence of the endogenous *cdx4* gene the ectopic expression of *cdx4* in the
anterior nervous system is not maintained (Fig. 4.3). This suggests that a Cdx4-dependent autoregulatory loop is necessary for the maintained cdx4 expression. FGF signaling is essential to this Cdx4 autoregulatory loop as evident by the similar lack of ectopic cdx4 expression in the absence of FGF signaling (Fig. 4.5). This is largely based on the fact that cdx4 nor hoxb trunk genes, although broadly induced, are maintained in regions outside of those that are known areas of FGF activity in the developing zebrafish nervous system (Fig. 4.1 & 4.4). Based on the importance of FGF and Cdx4 on the Cdx4 autoregulatory loop, I propose that the establishment and sustainment of this regulatory loop is essential to the maintenance of hox gene expression.

**New model of interaction between FGF, Cdx, and hox genes**

The results of this dissertation as a whole suggest a new paradigm of hox regulation by Cdx and FGF. The previous linear model (Fig. 1.3), although not wrong, leaves out key elements of hox regulation by Cdx and FGF. FGF may be an upstream signal of Cdx, but it is either not the only signal, or the loss of FGF signaling is compensated for by some other signaling cascade (Fig. 1.3). Cdx regulation by other signaling pathways, such as RA and Wnt, that play key roles in establishing the A-P axis of vertebrates has been shown. It seems that the most essential component to the regulation of hox genes in the developing posterior nervous system is Cdx, who works via direct and indirect mechanisms to regulate hox gene expression through all three phases. FGF’s essential role
is not inducing Cdx expression and activity, but instead FGF is necessary to maintain Cdx autoregulation (Fig. 1.3), which poses as the limiting factor in the direct and indirect roles of Cdx on *hox* gene regulation.
REFERENCES


Liu, A., Losos, K., Joyner, A.L., 1999. FGF8 can activate Gbx2 and transform regions of the rostral mouse brain into a hindbrain fate. Development 126, 4827-4838.


Spemann, H., Mangold, H., 1924. Über die Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. 100, 599-638.


