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CDX4 REGULATES THE DEVELOPMENT OF NEURAL CREST CELLS IN THE POSTERIOR BODY OF ZEBRAFISH EMBRYOS

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MANUEL ROCHA

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For my friends and family.

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ABSTRACT

The neural crest (NC) is a transient multipotent cell population that gives rise to a remarkable array of cell types, including cartilage, sensory neurons and glia, and pigment cells. NC cell development is a complex, multistep process that begins with induction and specification in the region between the neural and non-neural plate, termed the neural plate border (NPB). Once specified, NC cells undergo an epithelial-to-mesenchymal transition and migrate extensively throughout the body to the sites where they then differentiate. The various steps of NC cell development are orchestrated by cell-extrinsic signals and gene-regulatory interactions that can be described by a gene regulatory network (GRN), which consists of a series of interlinked modules. To date, the interrogation of the genetic mechanisms that govern NC cell development has largely focused on cells originating in the cranial region, despite our knowledge that the NC is regionalized into distinct subpopulations along the anteroposterior (AP) axis. In fact, it has been proposed that differences in the underlying genetic circuits account for the unique properties of cranial and trunk NC cells, namely their distinct differentiation potential and cellular behaviors during migration. Thus, a more detailed understanding of the mechanisms that govern the development of NC cells in the posterior body remains an important unaddressed question in the field.

In this thesis, I investigate the role of *cdx4* in regulating the development of NC cells in the posterior body of zebrafish embryos. Cdx factors are homeodomain-containing transcription factors that play a conserved and well-established function in the formation and patterning of the posterior body across vertebrate model systems. Furthermore, Cdx factors promote the formation of the spinal cord in both mouse and zebrafish. In this thesis, I demonstrate that *cdx4* is expressed in NC cell progenitors in the trunk and tail and is therefore a promising candidate for mediating the formation of NC cells in the posterior body.

In the course of my thesis work, I employed transgenic and genetic approaches in zebrafish embryos, together with high-resolution microscopy and bioinformatics analysis to uncover the

function of *cdx4* within the NC GRN and its role in establishing the trunk-specific behaviors during NC cell migration. In Chapter 2, I explore how *cdx4* links NC cell specification with the outgrowth of the posterior body. I demonstrate that Cdx4 binds near many of the genes that drive the early steps of NC cell specification, termed the NPB module. In particular, I demonstrate that *cdx4* is necessary for the expression of the NC specifier gene *foxd3*, in the posterior neural tube and the developing tailbud. Additionally, my analysis of available ChIP-seq and microarray data suggests that Cdx4 may regulate the signaling pathways and genetic circuits that drive posterior body outgrowth. In Chapter 3, I show that *cdx4* is necessary for the migration of trunk NC cells along segmental chains. Using *in vivo* single-plane illumination microscopy, I reveal that *cdx4* regulates the establishment of leader and follower identities that drive the directed migration of trunk NC cells. Further, the defects in trunk NC migration result in impaired development of the NC-derived sensory neurons that make up the dorsal root ganglia, which typically form as reiterated, segmental units. Finally, because *cdx4* expression and function is not limited to the NC, I performed cell transplantation experiments that demonstrated the impaired trunk NC migration is not due to loss of Cdx4 in the adjacent somitic mesoderm.

The results I present in this thesis suggest that *cdx4* functions as an early NC cell specifier in the posterior body. In particular, these findings support the assertion that Cdx4 regulates the expression of other early NC specifiers, including the genes of the NPB module, in the trunk and tail concurrent with posterior outgrowth. Further, I conclude that *cdx4* functions in premigratory NC cells to establish the leader and follower identities necessary for segmental migration. In summary, my thesis work has made significant progress towards establishing the genetic mechanisms that govern the development of NC cells in the posterior body of the zebrafish.

CHAPTER 1

INTRODUCTION

Attibutions

Much of the following introductory chapter was previously published in Rocha, M., Beiriger, A., Kushkowski, E. E., Miyashita, T., Singh, N., Venkataraman, V., and Prince, V. E. (2020). From head to tail: regionalization of the neural crest. Development, 147(20) [288] or Rocha, M., Singh, N., Ahsan, K., Beiriger, A., and Prince, V. E. (2020). Neural crest development: insights from the zebrafish. Developmental Dynamics, 249(1), 88-111 [289]. I here acknowledge the important contributions of my co-authors to those articles and thus to aspects of the text that follows.

1.1 Introduction

The neural crest (NC) is a transient, multipotent cell population characterized by its extensive migratory capacity and remarkable differentiation potential. NC cells give rise to a wide variety of derivatives, including neurons and glia of the peripheral nervous system, melanocytes, and craniofacial cartilage and bone [169]. Together with ectodermal placodes, cranial NC cells were key to the evolution of vertebrate-specific cranial elements, including a hinged jaw, special sense organs, and novel neural structures. These features are lacking in the protochordates (non-vertebrate members of the phylum Chordata), leading Gans and Northcutt to hypothesize that the vertebrate-specific cranial elements could be viewed as a "new head" [101, 236]. It was the evolution of the specialized vertebrate head, with its bony skull protecting a big brain, that facilitated the shift from passive to active feeding behaviors and enabled the extraordinary radiation of the vertebrate lineage [116].

Originally identified in chick embryos by Wilhelm His in 1868 (Hall, 2000), the NC has fascinated developmental biologists for over a century. In 1893 Julia Platt suggested the NC

origins of the skull based on research in the mudpuppy, a common salamander species [273]. Platt's work was highly contentious at the time because it challenged the dogma—based on germ-layer theory—that bone and cartilage must be of mesodermal origin. Her findings were nevertheless built upon by numerous subsequent researchers, in a series of reports that were summarized in a comprehensive and influential review from Sven Hörstadius [134]. In particular, Hörstadius provided a summary of work from his own group—primarily by his student Sellman—which used the classical embryology approaches of ablation and transplantation in amphibians to begin to reveal the remarkable migratory capacity of the NC and its broad contributions.

The development of the quail-chick chimera system in the early 1970s by Nicole Le Douarin (see Section 1.6) allowed for comprehensive analyses of NC migration and contributions. This system took advantage of the fact that the embryos of these closely-related avian species are of a similar size during the early stages of development, yet their cells exhibit unique nuclear morphologies. Because the nuclei of quail cells show a large mass of condensed heterochromatin upon Feulgen-Rossenbeck staining, researchers could use them as natural, indelible lineage tracers [168, 341]. By generating quail-chick chimeras, Le Douarin and colleagues (summarized in [166]) and Noden [251, 252, 253] elucidated NC cell migration pathways and derivatives along the anteroposterior (AP) axis. Initial reports by Raible and colleagues (1992) [283] and Schilling and Kimmel (1994) [306] described the development of NC cells in the trunk and head of zebrafish embryos, respectively. These foundational studies revealed that the basic organization of zebrafish NC cell migratory routes, and the array of derivatives the NC cells produce, are conserved between the teleost zebrafish and other vertebrates (Figure 1.1). Briefly, specific NC cell populations develop along the AP axis of the developing embryo, with the cranial NC population—the most anterior midbrain and hindbrain derived NC cells—contributing to the cartilaginous and bony elements of the jaw and skull, as well as producing neurons and melanocytes [147, 306, 367]. The trunk

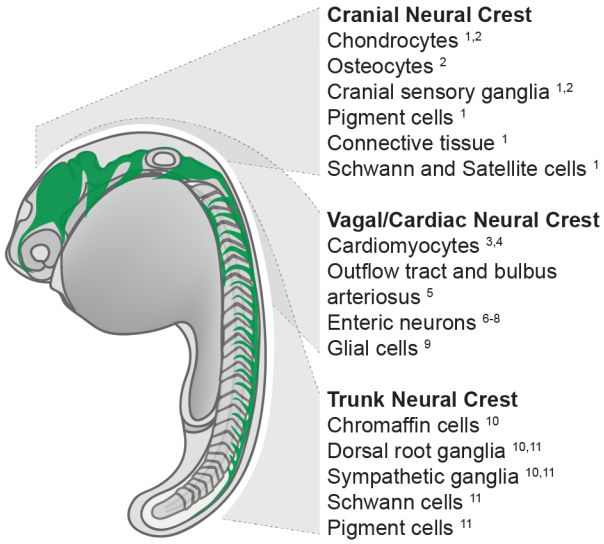

Figure 1.1: **Zebrafish neural crest regionalization and derivatives.** Schematic summarizing neural crest migration pathways (green) in the 22 hpf zebrafish embryo. Cell fates derived from specific anteroposterior levels are indicated. (1) Schilling and Kimmel (2) Kague et al. (3) Li et al. (4) Sato and Yost (5) Cavanaugh et al. (6) Elworthy et al. (7) Olden et al. (8) Shepherd et al. (9) Shepherd and Eisen (10) An et al. and (11) Raible et al.

NC population, which contributes neurons and glia to the dorsal root and parasympathetic ganglia, as well as melanocytes and other cell types, arises along the entire extent of the developing spinal cord, immediately adjacent to the developing mesodermal somites (Figure 1.1) [282, 283].

Based on these and other studies, we now understand that the NC is regionalized along the AP axis into discrete subpopulations with distinct differentiation potential: cranial, vagal, trunk, and sacral. Understanding the evolutionary and developmental origin of NC regionalization is essential, given that defects in NC formation that affect specific regional populations may lead to devastating diseases, such as Treacher Collins syndrome [354] or Hirschsprung's disease [20, 35]. However, the broad question of how the NC is regionalized into distinct cell populations along the AP axis remains a critical topic for further investigation.

Here, I will first introduce the molecular basis of NC cell development, focusing largely on

the key findings from studies using the teleost zebrafish, *Danio rerio*, as a model system. I will then explore the topic of NC regionalization along the AP axis, highlighting the differences in differentiation potential, underlying gene regulatory circuits, and migratory behaviors between these distinct cell populations. Finally, I will describe the Caudal-related family of homeodomain transcription factors, and detail evidence that supports the overarching hypothesis of my thesis, that Cdx factors may play a role in patterning the NC along the AP axis.

1.2 NC Induction and Specification

Induction, specification, and the onset of migration of NC cells occur in concert with the morphogenetic processes of gastrulation and neurulation. In zebrafish and other teleosts, neurulation processes differ markedly from those observed in the sarcopterygian models. Thus, it is important to understand teleost-specific features of neurulation, and to consider how these impact NC development.

1.2.1 Zebrafish neurulation

In sarcopterygians—e.g. chick, mice, *Xenopus*—the majority of the epithelial neural plate undergoes "primary neurulation", in which it folds or 'rolls up,' such that the lateral edges rise up and come together dorsally to form an epithelial tube with a central lumen. By contrast, the zebrafish neural plate is not strictly epithelial, nor does it fold [263, 307]. Early descriptions of zebrafish neurulation referred to the process as a form of "secondary neurulation" [263], a process limited exclusively to the most posterior neural tissue of sarcopterygians, which involves mesenchymal cells coalescing to produce a neural rod that subsequently cavitates to establish the lumen. However, secondary neurulation mechanisms also differ significantly from the processes of zebrafish neurulation, which involve pseudoepithelial cells and the organized establishment of a midline that allows the lumen to form. I

therefore concur with those authors who suggest the term secondary neurulation be avoided in the context of zebrafish and other teleosts [189].

Zebrafish neurulation begins at the tailbud stage (10 hours post fertilization; hpf) when gastrulation is reaching completion, at this point the zebrafish neural plate is a multilayered structure: in the anterior regions—fated to become fore, mid and hindbrain—the plate is 3-6 cells deep, thinning down to a single cell layer more posteriorly in the presumptive spinal cord [51, 130]. As development proceeds, neural plate cells converge, with lateral neural ectoderm cells moving towards the midline and intercalating while the most medial neural cells internalize and move ventrally. Together, these movements produce the neural keel, and subsequently the neural rod (Figure 1.2). It is at these same stages that molecular markers of NC progenitors are first expressed, implying that the first NC cells are specified as early as 11 hpf at the lateral edges of the anterior neural ectoderm [256, 352]. As neurulation continues, neural ectoderm cells produce a pseudostratified epithelium, with the cells undergoing polarized cell divisions to establish a well-defined midline by 18 hpf [31, 49, 51, 343]. Cells on either side of the midline then pull apart to produce the lumen of the neural tube, which despite its divergent mode of morphogenesis is now essentially equivalent to the neural tubes of other vertebrates.

Zebrafish NC cells begin to migrate away from the neural ectoderm in the cranial region by 14 hpf when the convergence processes of neurulation are still ongoing, although NC cells will also continue to emerge from the dorsal-most neural tissue after the neural tube is fully formed [21, 144, 306]. Given the dynamic morphogenesis that occurs during neurulation, the earliest NC cells derive from cells that lie in lateral positions, with only the later NC cells deriving from the dorsal-most aspect of the neural tube. As in other vertebrates, there is also an AP progression of NC cell migration, such that the first cranial NC cells begin migration about two hours earlier than the most anterior trunk NC cells, with the remaining trunk NC cells commencing their migration in a distinct AP progression.

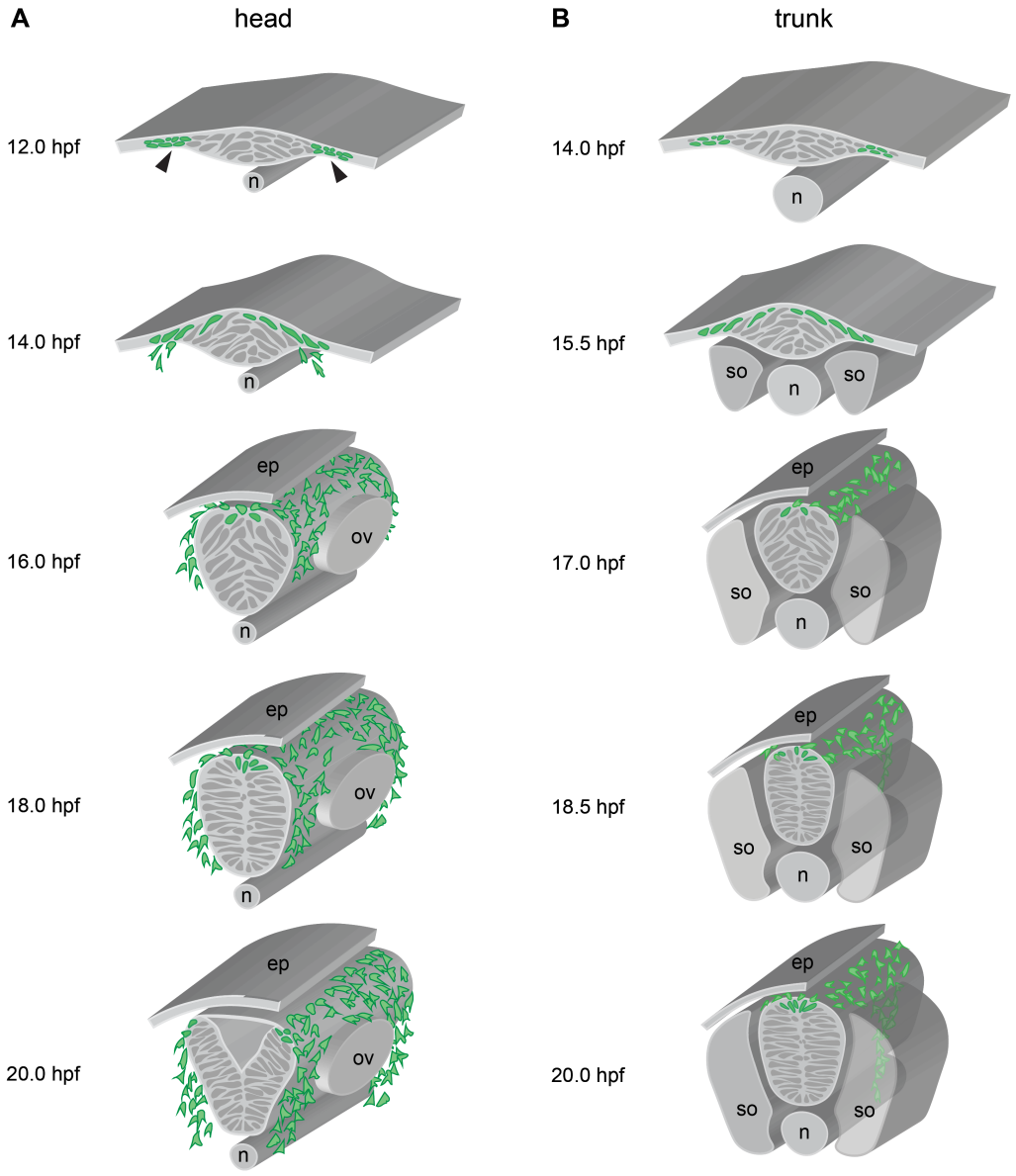

Figure 1.2: **Zebrafish neurulation and neural crest migration pathways.** A, Cranial neurulation and neural crest migration, schematized at the rhombomere (r)4-6 level, showing neural crest cells (green) migrating anterior and posterior of the otic vesicle towards pharyngeal arch (PA)2 and PA3, respectively. Stages are indicated, with laterally segregated cells apparent at 12 hpf (arrowheads), convergence movements forming the neural keel by 14 hpf, a neural rod at 18 hpf (with a clear midline now established), and finally a neural tube at 20 hpf. Note that the anterior terminus of the notochord lies under r4 in the zebrafish. B, Trunk neurulation and neural crest migration, schematized at the somite 7/8 level, showing neural crest cells (green) migrating on the medial pathway adjacent to the center of each somite. Stages are indicated. n = notochord; ep = epidermis; ov = otic vesicle; s = somite.

1.2.2 Regulation of NC induction

During gastrulation, the NC, and in the cranial region the adjacent placodal cells, are induced at the interface between neural and non-neural ectoderm, a finding initially made in axolotls [233, 234]. In chick embryos, this process is largely dependent on Bmp4 and Bmp7 signaling, with both Bmps expressed at the edge of the neural plate [184]. In the zebrafish, the *bmp2b*, *bmp4*, and *bmp7* genes are expressed on the ventral side of the embryo during gastrulation and, in concert with the action of the dorsally-localized BMP antagonist Chordin, establish functional gradients that play key roles in patterning both the mesoderm and the ectoderm along the dorsoventral (DV) axis [120, 249]. An intermediate level of BMP signaling induces NC fate [312], and altering the levels of BMP activity during gastrulation disrupts NC cell formation [241, 246]. In addition, Wnt/β-catenin signaling plays a role in NC induction, in part via regulation of expression of *foxd3* and *sox10* [180]. However, there is unlikely an absolute requirement for Wnt signaling in NC induction or subsequent development, as some markers of NC cells and their chondrogenic and pigment cell derivatives are still expressed when a dominant inhibitor of Wnt signaling is induced at the onset of migration [180].

1.2.3 Regulation of NC specification

In recent years, advances in molecular biology, and particularly next generation sequencing approaches, have fundamentally shaped our understanding of the gene regulatory interactions that underlie different events in NC development. This has led to the concept of a NC gene regulatory network (GRN), or rather a series of interlinked GRNs, that underlie the process of NC formation [219, 300, 324]. While the GRN has proven an invaluable tool, it is largely based on the integration of data from a variety of species. To appreciate the aspects of NC development that are either conserved or variable across species, I here provide a zebrafish-specific NC GRN based upon published data (Figure 1.3). This is not an exhaustive summary, but is designed to initiate the process of organizing relevant interactions.

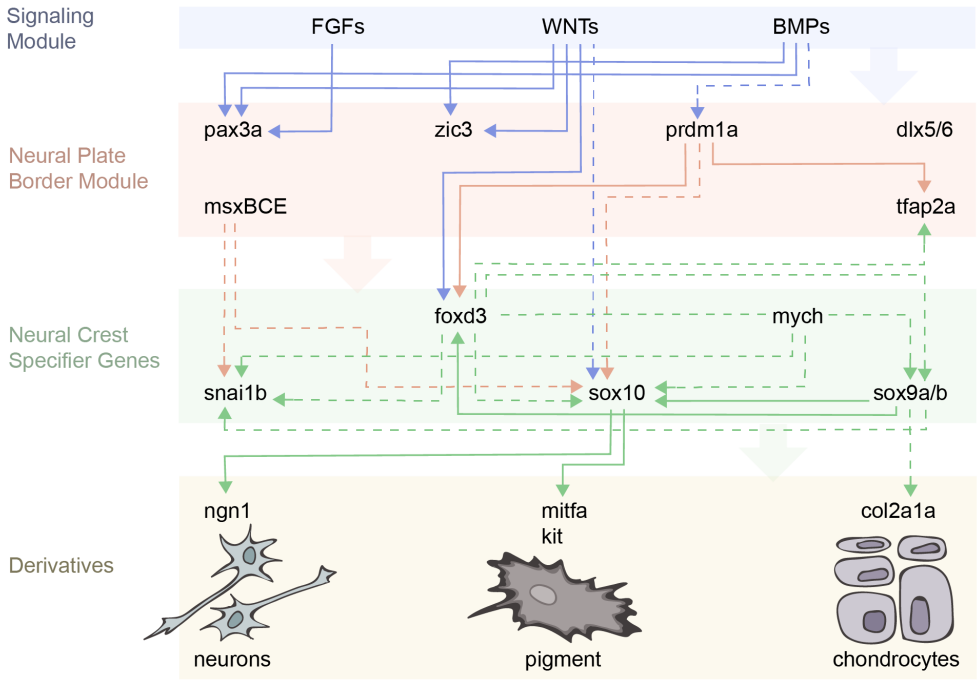

Figure 1.3: **A zebrafish-specific neural crest GRN.** This simplified gene regulatory network is built exclusively from zebrafish data; see text for details. Direct interactions are depicted with solid lines, whereas dashed lines show interactions inferred from loss-of-function studies.

According to the hierarchy of the NC GRN [300, 324] inductive interactions between neural and non-neural ectoderm specify the NPB by driving the expression of a battery of transcription factor genes. In the zebrafish (Figure 1.3) these include *pax3a* [103, 316], *tfap2a* [15, 159, 160], *msxB/C/E* [269], *zic3* [103], *dlx5/6* [240], and *prdm1a* [10, 26, 124, 259, 275, 294]. Three signals—BMP, Wnt, and FGF—are required for expression of *pax3a* at the NPB, whereas Wnt and intermediate levels of BMP are sufficient for *zic3* expression [103]. Across vertebrate species, these signals are integrated through evolutionarily conserved enhancers that respond to particular combinations of signaling inputs [103].

Cells at the NPB become specified to the NC fate as gastrulation draws to a close and segmentation begins, around 11 hpf. At this stage, several key transcription factor genes referred to as "NC specifiers" commence expression. These include *foxd3* [187, 229, 256, 334], *snai1b* [352], *sox10* [40, 78, 150], and *sox9a/b* [181, 381, 382]. Additionally, *twist* [63, 105], *mych* [131], and *id3* [72], all of which have been shown to play key roles in NC specification in other species [291, 324], are expressed in zebrafish premigratory NC cells. In the zebrafish, these latter three genes play a role in ectomesenchymal differentiation, survival, and proliferation, respectively. However, their role in NC specification has not been formally demonstrated. As the hierarchical GRN model predicts, loss of upstream NPB gene function impedes NC specification. For example, two different mutant alleles of *prdm1a*—*narrowminded* and *ubo*—display reduced NC cell numbers and no Rohon-Beard cells (RBs), a transient population of dorsal sensory neurons [10, 294]. Prdm1a directly activates expression of the NC specifier genes *foxd3* and *tfap2* [275], and also regulates the expression of both the NC specifier *sox10* and the RB marker *islet1* [259]. In MsxBCE morphants, expression of *snai1b* is lost and *sox10* reduced, yet the expression of *foxd3* remains unaffected [269]. Together these results indicate that the NPB genes activate expression of NC specifier genes through both specific and complex regulatory interactions.

Strikingly, phenotypic analysis of presumptive loss-of-function mutants in NPB and NC

specifier genes has revealed that these transcription factors are required for the development of both distinct and overlapping NC cell subpopulations. For example, *colourless/sox10* mutants have defects in NC-derived melanoblasts, neurons, and glia, while skeletal derivatives remain unaffected [78, 150]. Further, the *tfap2a/lockjaw* mutant displays defects in NC specification and migration, as well as in both chondrogenic and pigment derivatives [159, 160]. In a second mutant allele of *tfap2a*, termed *mont blanc*, defects in NC induction, specification, and even migration were not noted, but these specimens again exhibit significant defects in chondrogenic differentiation [15]. A more dramatic phenotype results from double morpholino knockdown of both Tfap2a and Tfap2c, which leads to the complete absence of NC cells [128, 182]. Similarly, both *sym1/foxd3* mutants [334] and Foxd3 morphants [187] exhibit defects in several NC cell derivatives, including peripheral neurons, glia, and cartilage, yet retain the proper number of melanocytes. On the other hand, mutants with the *mother superior* allele of *foxd3*, which disrupts a NC-specific regulatory element, exhibit a depletion of all NC cell derivatives that is preceded by a reduction in expression of *snail1b*, *sox9b*, and *sox10* [229]. Finally, analysis of *foxd3* and *tfap2a* double mutants has revealed that these embryos fail to express *snai1b*, *sox9a/b* and *sox10* and lack all NC cell derivatives [9, 370]. In summary, mutations of individual zebrafish NC specifier genes are often insufficient to block NC specification. Instead, experiments to date have revealed a robust combinatorial code that plays complex roles in both NC specification and subsequent cell fate decisions.

1.3 The epithelial-to-mesenchymal transition

Following induction and specification of NC, the NC cells must pass through an epithelial-to-mesenchymal transition (EMT), during which their morphology, adhesive properties, polarity, and behavior change dramatically as they transition into actively migrating mesenchymal cells [349]. EMT occurs in multiple contexts, and its roles in collective cell migrations, mor-

phogenesis, and cancer have been broadly investigated [91, 92, 99, 175, 353].

The process of EMT is complex, involving changes in cell adhesion, cell-cell, and cell-matrix interactions, as well as the extension of protrusions such as filopodia and pseudopodia [21, 85, 117, 301, 302, 351]. This morphological transition requires the regulation of many effector genes [324]. Critically, the GRN that underlies NC EMT in sarcopterygian models includes several transcription factors also involved in NC fate specification, such as FoxD3, SoxE and Pax3/7, as well as Snail1/2 and Twist [324]. In zebrafish, the transcriptional regulation of EMT has not yet been investigated in detail. Zebrafish *snai1b* and *twist1a/b* are expressed in NC cells at the time of EMT, however their role in this process has not been formally evaluated [63, 105, 144, 352].

Although the molecular underpinnings of zebrafish EMT remain incompletely understood, our understanding of the cellular basis of EMT has benefited from live imaging approaches available in the zebrafish model. In particular, a series of studies from the Halloran lab has provided detailed insights into EMT of the zebrafish cranial NC cells. An initial study used single cell labeling to reveal that the earliest cranial NC cells originate from lateral to, or overlaying, the developing neuroepithelium at the 13-15 hpf stage, with later NC cells arising at 15-19 hpf from the pseudostratified neuroepithelium [21]. This recognition of two separate populations of cranial NC cells is consistent with the initial morphological description of the region [306], and with follow up studies using transgenic markers [144].

Subsequent detailed analyses showed that the later migrating NC cell population initiates EMT at the point of apical detachment from the midline of the neuroepithelium (Figure 1.4) [21, 52], when activation of the small GTPase Rho becomes restricted to the apical region by the RhoGAP Arhgap1, leading to F-actin enrichment [53]. Next, the NC cells round up and form membrane-based blebs—rounded bulges on one side of the cell—and then begin to extend filopodial and lamellipodial protrusions as they exit the neuroepithelium and migrate away [5, 21, 52]. Interestingly, blebs and filopodia exhibit different underlying actin

localization and dynamics, consistent with an ongoing series of morphological transitions [21]. Bleb formation is crucial for EMT to progress, as inhibition of Myosin-II or Rho-kinase (ROCK), which disrupt blebbing, inhibits EMT [21]. Recent work from the Prince lab has also implicated Prickle1a and Prickle1b—core Planar Cell Polarity (PCP) molecules—in zebrafish cranial NC cell EMT. While in wild type embryos the blebbing phase of EMT typically completes within 20 minutes, in Prickle1-deficient embryos the majority of NC cells are delayed in the transition from blebbing to mesenchymal morphologies [5], indicating a block in the EMT process.

EMT also involves cell surface changes and the dissolution of cadherin-mediated adherens junctions. Initially, evidence from several model species indicated that NC cells undergo a "cadherin switch" from E-cadherin to N-cadherin during EMT [339, 340]. Consistent with these findings, in zebrafish the premigratory NC cells predominantly express E-cadherin (Cdh1), whereas migratory NC cells predominantly express N-cadherin (Cdh2) [5, 305]. E-cadherin represses contact inhibition of locomotion (CIL) by controlling protrusions via Rac1 and p120 [305]. N-cadherin is expressed in migratory NC cells, where it plays a critical role in promoting migration [271, 276]. The proper cellular localization of N-cadherin is regulated by several inputs associated with Shh and Wnt signaling, and disruption of these inputs causes NC cell migration to stall. For example, mutant analysis has revealed that the putative Shh receptor Cdon localizes N-cadherin to allow directed migration of trunk NC cells [276]. Similarly, deficiencies in Rabconnectin-3a and its associated v-ATPase subunit lead to misregulation of cadherins by disrupting vesicle endocytosis and Wnt signaling, resulting in a failure of NC cells to migrate—with phenotypic analysis in this study focusing on the cranial region [358]. Further, disruption of the Wnt target gene Ovo1 similarly disrupts intracellular trafficking, thus blocking localization of N-cadherin at the membrane and again disrupting cranial NC cell migration [271]. Interestingly, Prickle1b-deficient embryos have elevated levels of E-Cad in both premigratory and migratory cranial NC cells, and decreased

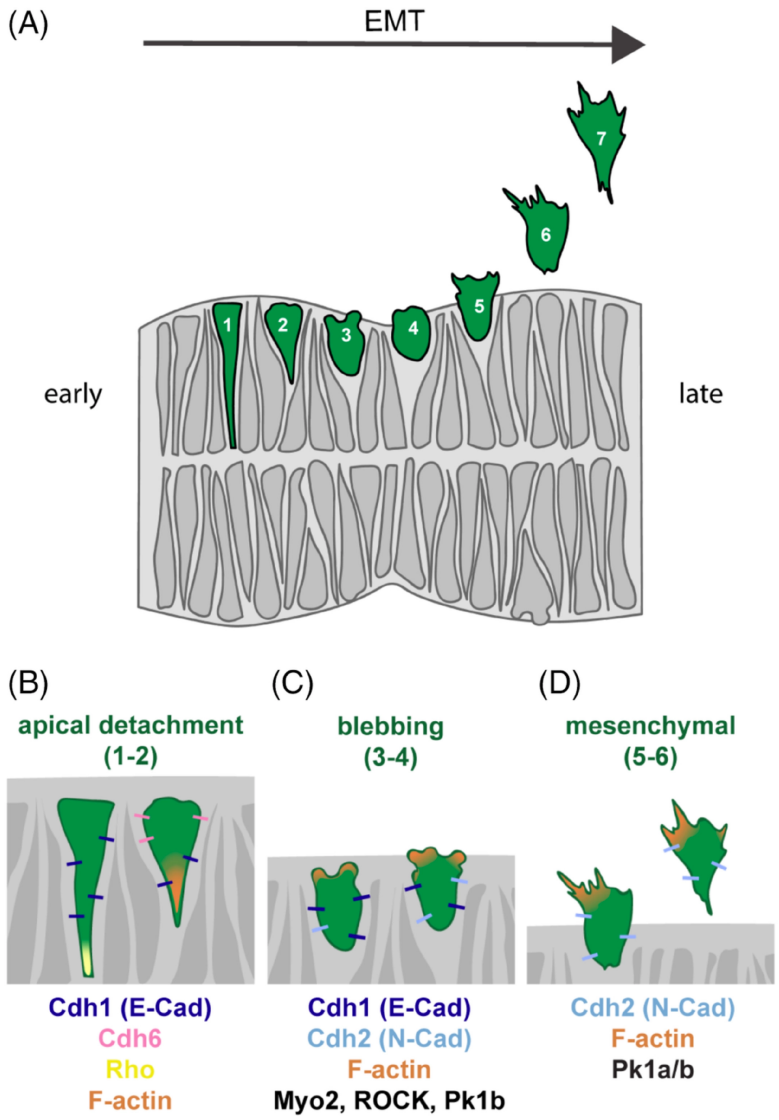

Figure 1.4: **Zebrafish neural crest cells undergoing the epithelial-to-mesenchymal transition.** Zebrafish neural crest cells undergoing the epithelial-to-mesenchymal transition. A, A single premigratory neural crest cell (1) is shown over successive time points (from left to right) undergoing apical detachment from the neuroepithelium (2), blebbing behavior (3-4), and ultimately becoming a fully mesenchymal and migratory neural crest cell (5-7). B, Molecular regulators of apical detachment, C, blebbing, and D, mesenchymal states; see text for details.

levels of N-Cad in the migratory cells, indicating a potential link between PCP and cadherin regulation [5].

Recent studies in other models have revealed a more complex and dynamic regulation of cadherins [340]. Briefly, a second switch from type I cadherins (E- and N-cadherin), which mediate strong cell-cell interactions, to more mesenchymal type II cadherins (including Cadherin-7 and Cadherin-11) also takes place [196, 204, 340]. In zebrafish, Cadherin-6 (Cdh6) is upregulated prior to EMT and promotes apical detachment by regulating the spatiotemporal dynamics of F-actin and active Rho GTPase [52]. On the other hand, the roles of Cadherin-7 and Cadherin-11 have not been investigated in zebrafish; however, given their roles in NC cell EMT in other models it is possible that the zebrafish homologs of these molecules will similarly prove to play important roles in the zebrafish NC.

1.4 NC Migration

After completing EMT, NC cells migrate collectively along stereotypic pathways, often traveling great distances before reaching their destination and differentiating into a variety of derivatives [28, 204]. Migration of NC cells is generally regulated by a combination of attractive and repulsive cues, as well as by cell-cell interactions [350]. Here, I summarize the prevailing models that explain how collective migration of NC cells is regulated.

1.4.1 Contact Inhibition of Locomotion

Several studies support a model whereby contact inhibition of locomotion [1] or "CIL"–based dispersion and mutual attraction promote the collective directional migration of NC cells [349, 350]. Research from the Mayor group showed that in *Xenopus* NC explants cells at the edge were polarized, projected large lamellopodia, and had higher persistence of migration than interior cells [38]. When two explanted migrating NC cells came into contact, they collapsed their protrusions and migrated away from each other, suggesting that CIL plays

a critical role in directional migration of NC cells [38]. In follow up studies, the molecular basis of CIL has also been investigated in some detail in *Xenopus*. These studies showed that the Wnt/planar cell polarity (PCP) pathway mediates CIL during NC migration, in part by promoting RhoA activation at the site of contact and Rac1 activity at the free edge in an N-cadherin-dependent manner [38, 67, 203, 348]. Further, the chemoattractant Sdf1 amplifies and stabilizes cell polarity, which results in directional migration [348]. Critically, *Xenopus* NC cells also exhibit mutual attraction mediated by the complement factor C3a and its receptor C3aR [39]. In supplement to comprehensive *Xenopus* explant experiments, live imaging of zebrafish embryos transgenic for the NC cell marker *sox10:gfp* demonstrated that migrating cranial NC cells also exhibit CIL *in vivo* [38]. While *syndecan4* [203] and *par3* [230] have been shown to play key roles in zebrafish NC migration and CIL, the degree to which the molecular mechanisms elucidated in *Xenopus* are conserved in zebrafish remains to be explored further.

1.4.2 Trailblazer model

Interestingly, mathematical models from the Kulesa lab show that simple chemotaxis is insufficient to explain the experimental observations of long-distance migration by NC cells [211]. On the other hand, when the model is refined to include two NC cell subpopulations—cells at the migratory front that respond to a VEGF gradient and late–emerging trailing cells that respond to directional cues from leading NC cells—it predicts that the majority of cells invade in a multicellular stream [211]. Moreover, transcriptional profiling of migrating chick cranial NC cells revealed that leading and trailing cells possess distinct gene expression profiles [211]: the leading cells show upregulation of genes involved in cell guidance and navigation, whereas the trailing cells show upregulation of distinct cadherins [211]. Additional profiling of single chick cranial NC cells uncovered a unique gene expression signature in a small fraction of lead cells termed "trailblazers" [212]. This signature includes 16 genes that are

up- or down-regulated, with the TGF-β signaling antagonist *BAMBI*, and the *CXCR1* and *NOTCH1* receptors, consistently expressed at high levels by trailblazers across two different phases of NC cell migration. Notably, exposure of NC cells to the chemoattractant VEGF *in vitro* upregulates a subset of genes associated with the lead cell signature, suggesting that it induces trailblazer cell identity [213].

1.5 NC Differentiation

The zebrafish has proven helpful to unpacking how intrinsic gene regulatory states interact with extrinsic environmental signals to mediate the differentiation of NC derivatives. Zebrafish NC cells are fate-restricted from an early stage, as individually labeled premigratory NC cells typically produce differentiated cells of only a single class [151, 283, 306]. Dorsky et al. (1988) [74] confirmed the initial report from Schilling and Kimmel (1994) [306] of clonal restrictions in cranial NC cells, and further showed that cell fates are biased by Wnt signaling, with medial Wnt signals promoting pigment cell fates. Further, clonal analysis revealed that those NC cells that do give rise to multiple kinds of derivatives likely do so by first forming type-restricted precursors [282]. These results suggest that NC cells are specified to their final fates well before they reach their ultimate locations.

Recently, single cell RNAseq of murine NC cells revealed that migrating NC cells undergo a series of sequential binary fate restrictions. Further, in situ sequencing showed that these distinct states show spatial segregation. Specifically, the first bifurcation separates the sensory lineage from the common progenitors of autonomic and mesenchymal fates, and the second split separates the autonomic neuronal fate from mesenchymal progenitors [330]. This analysis is especially valuable because it elucidates the transcriptional regulation of cell fate decisions. Namely, each decision consists of an initial coactivation phase, where genes of competing cell fate programs are coexpressed, followed by a gradual biasing towards a particular fate, and finally a commitment phase where mutually exclusive, fate-specific gene

expression programs becomes activated.

In addition to intrinsic regulatory programs, proper migration and environmental signals are also necessary for NC cell differentiation. For example, *erbB3* mutants lack DRG not because of an inability to specify neurons, but rather because NC cells fail to pause at the location where DRGs should form and thus do not receive the necessary signals to differentiate [132]. A great deal of research has been published on the differentiation of the various NC cell types—below, I present a brief discussion of zebrafish NC cell differentiation into three specific cell types: pigment cells, chondrocytes, and neurons.

1.5.1 Pigment Cells

Zebrafish are an invaluable model for the study of pigment development and pattern formation due to the wealth of pigment mutants identified during the large-scale mutagenesis screens of the 1990s [149, 255]. However, as several recent reviews have already covered this topic in significant depth [56, 140, 284], I touch on only a few highlights in the section below.

Zebrafish NC cells give rise to three major pigment cell types—yellow xanthophores, iridescent iridophores, and black melanocytes [284]. Melanocyte precursors begin expressing melanin pigment around 24 hpf and the embryonic melanocyte pattern is largely established by 48 hpf [284]. During larval-to-adult metamorphosis, this pattern is gradually replaced by the adult pigment pattern [145]. Importantly, the adult pigment cells are largely derived from post-embryonic melanocyte stem cells (MSCs) that originate from the NC [32, 139, 357]. These MSCs are established within the first 2 days and remain closely associated with peripheral nerves and ganglia, including the DRG, which likely provide a niche [32, 73]. Further, the developmental mechanisms of embryonic and metamorphic pigment cells may be decoupled, as in *puma* (*tuba8l3*) mutants that lack adult melanophores while embryonic melanophores remain unaffected [264, 265].

As in other species, the basic helix–loop–helix/leucine zipper transcription factor Mitfa

plays a central role in melanocyte differentiation [186]. Melanophores are absent throughout embryonic and larval development in *nacre/mitfa* mutants, a phenotype which persists through adulthood. On the other hand, the mutants show an increase in the number of iridophores. The *mitfa* gene functions cell-autonomously in the melanophores, where it is required for the expression of *trp2* and *kit* [186]. Analysis of the *mitfa* promoter has revealed Tcf/Lef binding sites [75], suggesting that Wnt signaling, which promotes pigment cell fate at the expense of neurons and glia [74], may do so by directly regulating *mitfa* expression [74, 75]. Moreover, the *mitfa* promoter contains Sox10 binding sites, which are necessary for its expression both *in vitro* and *in vivo* and expression of *mitfa* is sufficient to rescue melanophore development in *sox10* mutant embryos [81].

The establishment of the adult pigment pattern requires cell-cell interactions between different pigment cell types [83, 95, 119, 194, 238, 267, 266, 366]. The genetic and molecular mechanisms by which the self-organizing stripe pattern is generated were recently reviewed in detail by Irion and colleagues (2016) [140]. An exciting recent finding from the Parichy lab is that macrophages are necessary to relay long-distance signals between xanthophores and melanophores via specialized cellular projections termed airinemes [84]. Whether airineme/macrophage-mediated signaling functions in other contexts remains to be explored.

1.5.2 Chondrocytes and cranial morphogenesis

The elaborated cranium, a key feature of all vertebrates [101], is largely derived from the cranial NC. In humans, defects in crest-derived, or crest-influenced, craniofacial structures are relatively common [204, 355]. Unsurprisingly, there is a great deal of interest in understanding the patterning and differentiation of the NC-derived cartilaginous and bony elements of the skull. Two recent reviews have highlighted the benefits of the zebrafish in this context [232, 364].

Foundational fate mapping of the zebrafish cranial NC was established by Schilling and Kimmel (1994) [306]. By dextran-labeling of individual cells, they not only revealed the AP regionalization of the cranial NC cells, but additionally showed that particular cell fates are influenced by the initial mediolateral position of NC cells. The most laterally-derived cells tend to differentiate into neurons of the cranial ganglia, whereas more medially located cells migrate longer distances into the pharyngeal arches to differentiate into cartilage. Other fates—glia and melanocyte derivatives——are less spatially restricted.

More recently, transgenic reporter lines have allowed detailed fate mapping of the cranial NC contribution to the anterodorsally-located neurocranium [76, 367]. These studies revealed that NC cells derived from the anterior midbrain migrate over the eye to contribute to the medially-located ethmoid plate of the neurocranium, whereas NC cells from the posterior midbrain migrate behind the eye to contribute to the bilateral trabeculae elements. The more posterior components of the neurocranium do not derive from the NC, but rather from the mesoderm. The precise interface between the NC–derived and mesoderm-derived elements was revealed via tracing experiments that used a tamoxifen-inducible Cre-based lineage marker driven by the *sox10* regulatory sequences [227]. The indelible nature of the marker allowed lineage tracing though to adult stages, revealing late NC contributions to additional cranial structures, including the gill pillar cells and the barbels, which are tentacle-like chemosensory structures. A variety of genetic manipulations have revealed significant conservation in the fundamental molecular mechanisms that pattern the zebrafish anterior neurocranium and the human hard palate [336], suggesting that zebrafish can provide a useful model of palate development [232].

Differentiation of NC cells into chondrocytes depends on gene regulatory interactions [324]. Studies in mice suggest that the NC specifier gene *Sox9* is a key regulator of chondrogenic fate, functioning in part by regulating the expression of extracellular matrix (ECM) components and genes encoding ECM modification enzymes [24, 257]. In zebrafish, muta-

tions in *jellyfish/sox9a* [381, 382] or *sox9b* [382] result in craniofacial defects and reduced expression of the collagen–encoding gene *col2a1a* [382]. Notably, the *sox9* paralogs play distinct roles during cartilage morphogenesis: *sox9a* is necessary for chondrocyte stacking, while *sox9b* mutants fail to attain proper number of chondrocytes [382]. Double mutants in both *sox9a* and *sox9b* completely lack both pharyngeal cartilage and the neurocranium, suggesting that the *sox9* paralogs have both overlapping and distinct functions during pharyngeal cartilage development [382].

The cranial NC cells carry some intrinsic information with them into the pharyngeal region [253, 250], however signals from the surrounding tissues, including Endothelin 1 [54, 157, 222, 223, 237], Shh [79, 367], and Bmp [6, 387] are necessary for proper formation and patterning of the skeletal elements in the pharyngeal arches [161, 214, 224]. Fgf signals are also critical for chondrocyte development, with early expression of Fgfs in the neural tube and lateral plate mesoderm, as well as later expression in the pharyngeal endoderm, influencing distinct aspects of pharyngeal cartilage development. If early Fgf signals are lost, the endodermal pouches of the pharyngeal arches fail to form, and subsequently, the pharyngeal cartilages are reduced or absent [61, 285, 286]. Later, Fgf signals from the pharyngeal endoderm are required for induction and survival of chondrogenic precursors [64, 368]. Another example of the importance of pharyngeal endoderm in patterning adjacent NC derivatives, is the requirement for endodermal Integrinα5 function in development of hyoid (second) arch derivatives [62]. Integrinα5 is necessary for development of the first endodermal "pouch", which in turn controls cartilage development from the adjacent NC cells, apparently by regulating not gene expression, but rather the compaction and survival of NC-derived chondrocytes.

Overall, the zebrafish has shown particular utility in allowing researchers to visualize chondrogenic precursors *in vivo* using transgenic tools, which when coupled with genetic manipulation can provide new insights into how signals are integrated to drive cartilage

development and morphogenesis. A powerful example of this approach uncovered the integration of Notch, Endothelin and Bmp signaling in the control of skeletal patterning in the upper face [16]. Further, a recent study has coupled quantitative measurements of gene expression, and live imaging, with a computational approach, to model the complexities of the signaling networks that pattern the mandibular (first) arch structures that give rise to the jaw [215]. Such detailed models not only provide new insights into zebrafish pattern formation, but have the potential to be extrapolated to other species and ultimately shed light on how widely differing jaw structures have evolved.

1.5.3 Neurons and Glia

DRG and Sympathetic Neurons

NC cells at all axial levels are capable of giving rise to neurogenic derivatives. In the cranial region, NC cells contribute to the cranial ganglia as well as to Schwann and satellite cells [147, 306]. In the trunk, NC cells give rise to sensory neurons of the dorsal root ganglia (DRG), autonomic sympathetic neurons, and Schwann cells [283, 282]. In zebrafish embryos, DRG neurons begin their differentiation at 36 hpf, about 20 hours after the onset of trunk NC migration [8]. In contrast, sympathetic neurons are not detected until at least 2 days after the differentiation of DRG neurons [8]. Individual DRGs and sympathetic ganglia initially contain only a few differentiated neurons [8]; however, the number of neurons progressively increases due to continued neuronal cell division until at least 4 weeks of age [8]. In this regard, the zebrafish is very different to chick and mouse embryos, in which neuronal differentiation is concomitant with terminal mitosis, and an early over-population of neurons is reduced via apoptosis [41, 201, 292]).

As in other model systems, zebrafish *sox10* plays a crucial role in the specification of neuronal lineages, as well as glia and pigment cells [40, 78, 150]. *sox10* mutants exhibit marked reduction of both DRG neurons and the associated Schwann cells and satellite glia

[40]. However, the observed reduction in DRG neurons is not due to cell death or changes in proliferation rates, and DRG survival is not dependent on proximity to glial cells. Instead, these mutants show reduced expression of the proneural gene *neurog1* and exhibit frequent gaps in its stereotyped segmental expression pattern [40]. Furthermore, Sox10 is transiently expressed in DRG sensory neuron progenitors, where it induces the expression of *neurog1* [40]. Neurog1, in turn, plays a key role in the formation of sensory neurons [40, 69, 207]. Interestingly, if *neurog1* function is lost, cells fated to become DRG neurons instead adopt a glial fate [207]. It is noteworthy that at earlier stages Neurog1 promotes RB sensory neuron fates, and at that point must be excluded from the NC cells; yet later, this same transcription factor acts within the NC cells to promote DRG fates.

The *baz1/sox10* mutant, in which a single nucleotide substitution impairs the DNA-binding HMG domain, exhibits features typical of other *sox10* mutant alleles but also displays supernumerary sensory neurons [40, 69]. The *baz1* allele retains the ability to drive expression of *neurog1* [69]. Further, overexpression of *neurog1* rescues the loss of sensory neurons in Sox10 morphant embryos [69], suggesting that the activation of *neurog1* by Sox10 is critical for the establishment of neuronal fate. Additionally, lateral inhibition likely plays an important role in the balanced production of neurons and glia during DRG formation. Consistent with this model, *notch1a*, *deltaA*, and *deltaD* are expressed in non-neuronal cells associated with the DRGs [208] and inhibition of Delta/Notch signaling results in an increased number of DRG neurons [208, 69].

Enteric Neurons

The enteric nervous system (ENS) of the zebrafish is derived entirely from the vagal NC (Figure 1.1), unlike in amniotes where the ENS arises from a combination of vagal and sacral NC cells [318]. The role of the ENS is to innervate the intestine to regulate various aspects of its function, including motility, secretion, and local blood flow [102]. In humans, improper

development of the ENS can result in devastating syndromes, such as Hirschsprung's disease where the distal portion of the intestinal tract lacks innervation and is thus unable to generate proper bowel movements [102]. The zebrafish has emerged as a useful model in which to interrogate ENS development.

Zebrafish ENS precursors enter the anterior portion of the digestive system at 32 hpf and migrate posteriorly along the length of the developing gut as two parallel chains, reaching its posterior end by 66 hpf [82, 258, 320]. The expression of *phox2b* in ENS precursors is conserved in zebrafish, where it is necessary for proper development [82]. Similarly, components of the GDNF/Ret signaling pathway are expressed in the subset of vagal NC cells that give rise to the ENS after the onset of migration towards the gut and play a crucial role in precursor migration [319, 320]. In zebrafish, *ret* is expressed as two isoforms, *ret9* and *ret51*, and the Ret9 isoform is sufficient for colonization of the gut by enteric neurons [123]. Moreover, when *gfra1a*/*gfra1b* or *ret* are knocked down, enteric NC cells are still capable of expressing *phox2b* and entering the anterior gut, indicating that their initial specification is not affected. However, they are not able to migrate posteriorly or increase in numbers, resulting in markedly fewer enteric neurons along the length of the gut [320]. These results, together with the fact that *sox10* mutants exhibit reduced numbers of ENS neurons [78, 150], suggest that aspects of ENS development are largely conserved across the vertebrates.

Olden and colleagues (2008) [258] proposed that ENS development can be divided into four distinct phases. At 32-50 hpf, all precursors express *crestin* prior to neural differentiation. At 55-58 hpf, anterior precursors begin neuronal differentiation as a group and downregulate *crestin*, while posterior precursors retain *crestin* expression, do not yet undergo differentiation, and proliferate at a higher rate [258]. At 62-69 hpf, anterior precursors no longer express *crestin*, while posterior precursors have begun downregulating its expression. Posterior precursors also begin to differentiate into neurons in an anterior to posterior wave. During this stage, proliferation rates are equal between anterior and posterior re-

gions. Finally, at 69-99 hpf, posterior precursors no longer express *crestin* and continue to differentiate throughout the intestine while the rate of proliferation decreases [258]. More recently, analysis of the spatial and temporal colocalization patterns of *phox2b*, *ret*, and *sox10* revealed three major ENS progenitor subpopulations that are indicative of distinct developmental states [344]. Namely, less mature progenitors at the wave front express all three genes, while more mature progenitors lose expression of *sox10* and/or *ret* [344].

Although several factors involved in ENS specification and development have been uncovered, the mechanisms that regulate enteric NC cell migration along the gut are less well understood. Because of their amenability for live imaging and experimental manipulations, zebrafish embryos are proving useful to probe this question *in vivo*. As an example, Uribe and colleagues demonstrated that Retinoic Acid (RA) is necessary for the migration and survival of enteric NC cells along the developing gut [361]. Specifically, RA functions during the time window when NC cells migrate into and along the foregut to maintain collective chain migration and ensure complete colonization of the gut [361]. This function is mediated at least in part by regulation of *ret* and *meis3*, genes previously shown to be required for colonization of the gut by NC cells [360].

1.6 Axial differences in NC differentiation potential

The pioneering lineage tracing experiments using the quail—chick chimera demonstrated that—at least in amniotes—only cranial NC cells give rise to ectomesenchymal derivatives, including cartilage, connective tissues, dermis, dermal bone, and teeth. By careful lineage tracing, Le Douarin and colleagues conclusively showed that cranial NC cells contribute to the facial and visceral skeleton and its adjacent connective tissue. Briefly, NC cells from the prosencephalon and the mesenecephalon form the nasal and periorbital skeleton and contribute to the cranial vault. Mesencephalic NC cells additionally give rise to the skeleton of the upper and lower jaws, the palate, and the tongue. They also contribute to the pre-otic

region, alongside rhombencephalic NC cells. Finally, cartilage of the hyoid and posterior pharyngeal arches is derived from rhombencephalic NC cells [166, 252]. Cranial NC cells also produce loose connective tissue of the lower jaw, tongue and ventrolateral part of the neck, as well as dermis and striated muscles of the branchial arches [166, 252].

More recent lineage tracing experiments revealed that the cranial NC migration pathways [191] and craniofacial derivatives [162] maintain the spatial organization of the rhombencephalon and mesencephalon from which they derive. Moreover, the contributions of cranial NC cells are influenced in part by the action of intrinsic factors, including the Hox genes. Hox genes play a critical role in patterning the skeletal derivatives of NC cells arising from the posterior rhombencephalon (rhombomeres (r)4-r8). By contrast, NC cells that arise from the prosencephalon, mesencephalon and anterior rhombencephalon (r1 and 2)—which form the bones of the cranial and facial skull—do not express Hox genes [58, 59, 60]. Accordingly, transplanting Hox-expressing neural folds from r4-5 into the anterior, Hox-negative domains [58], or ectopically expressing Hox genes in the diencephalic neural folds [60], caused defects in the lower jaw and facial skeleton. Thus, Hox expression is incompatible with proper development of the jaw or facial derivatives of NC cells.

In addition to the intrinsic function of Hox genes, extrinsic signals from the surrounding tissues are instructive in the development of cranial NC skeletal derivatives. This was elegantly demonstrated by experimental manipulations of the chick foregut endoderm. When researchers ablated strips of foregut endoderm, specific cranial NC-derived skeletal structures failed to develop, while grafts of ectopic foregut endoderm altered the identity of the skeletal structures [60]. Importantly, only anterior, Hox-negative NC cells can respond to these endoderm-derived cues, whereas posterior, Hox-expressing NC cells do not form bone and cartilage in response to anterior foregut endoderm grafts.

Strikingly, these studies indicated that the posterior limit of skeletogenic NC cells corresponds to the level of the 5^{th} somite [172], near the transition between the rhombencephalon

and the spinal cord. To determine whether this represents an intrinsic feature of the cranial NC or results from different signaling environments, Le Douarin and colleagues performed a series of transplantation experiments (summarized in Figure 1.5). When quail mesencephalic and anterior rhombencephalic primordia were grafted into the chick neural axis at the level of somites 18-24, donor quail cells differentiated into dermis, cartilage, and connective tissues [171], suggesting that cranial NC are still capable of generating ectomesenchymal derivatives in an ectopic environment. Conversely, bilateral grafting of the trunk NC primordium into the anterior rhombencephalon resulted in the absence of facial and branchial skeletal elements [167, 239]. Similarly, when trunk dorsal neural tube was grafted to the midbrain, donor NC cells failed to form normal corneal derivatives, contributed fewer neurons to the trigeminal ganglion, and did not form cartilage even when grafted directly to the first branchial arch [193]. These results demonstrate that chondrogenic potential is an intrinsic and distinguishing feature of cranial, but not trunk, NC.

Nevertheless, the signaling environment is also important in directing ectomesenchymal differentiation. Cranial and trunk NC cells differ in their survival and differentiation in response to various extracellular signals *in vitro* [4]. Yet, when quail trunk NC fragments are unilaterally grafted to the anterior rhombencephalon of a chick host, donor NC cells migrate alongside host NC cells. In these chimaeras, quail ectomesenchyme derivatives are detected in connective tissues, dermis and muscle, but not cartilage or bone (Figure 1.5) [239]. These results suggest that the host cranial NC might provide extrinsic signals that allow trunk NC cells to give rise to a subset of ectomesenchymal derivatives. Moreover, when avian trunk NC cells are cultured in media commonly used for growing bone and cartilage cells, they generate ectomesenchymal derivatives *in vitro* [55, 206] and contribute to cranial skeletal components when transplanted into the head [206]. Thus, while chondrogenic potential is an intrinsic feature of the cranial NC, the signaling environment contributes to promoting this fate.

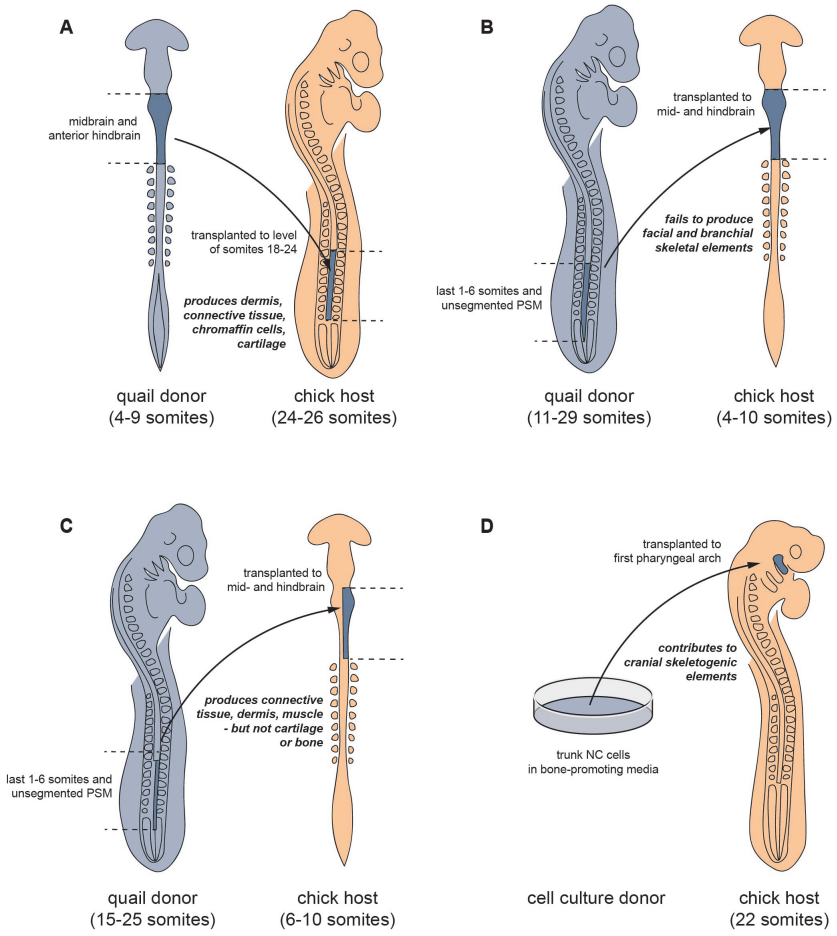

Figure 1.5: **Transplantation approaches reveal that intrinsic factors and extrinsic signals underlie differences between cranial and trunk NC cells.** Transplantation experiments reveal differences in the contributions of cranial and trunk neural crest. Quail embryos are shown in blue, chick embryos in orange. (A) Bilateral and heterotopic transplant of cranial (midbrain and anterior hindbrain) neural primordium from a quail donor (4-9 somite stage; ss) to the trunk of a chick host (24-26 ss) leads to the formation of skeletogenic derivatives, as well as chromaffin cells, at ectopic posterior positions. (B) The reciprocal transplant of trunk neural tube from a quail donor (11-29 ss) to the cranial (mid- and hindbrain) region of a chick host (4-10 ss) shows that trunk NC does not form skeletogenic derivatives. (C) A unilateral version of the transplant experiment shown in B demonstrates that host tissue can influence the migration and potential of transplanted cells. Donor cells form connective tissues alongside the host NC but cannot formskeletogenic derivatives. (D) When trunk NC cells cultured in bone-promoting media are transplanted into the mandibular and maxillary primordium of a chick host, the transplanted cells are able to form skeletogenic derivatives, demonstrating the importance of the NC signaling environment for cell fate decisions.

While much emphasis has been placed on the development of the cranial NC, it should be noted that trunk NC cells also give rise to unique cell types and exhibit distinct cellular behaviors. At the level of somites 18-24 in the chick, some NC cells form chromaffin cells, the neuroendocrine cells of the adrenal medulla [166]. It has commonly been held that sympathetic neurons and chromaffin cells are derived from a common lineage of catecholaminergic NC-derived progenitors, termed sympathoadrenal progenitors, that migrate to the dorsal aorta (reviewed in [138]). However, recent lineage tracing and genetic ablation experiments have revealed that chromaffin cells are, in fact, largely generated by Schwann cell precursors, a NC-derived population of peripheral glial progenitors that migrate along motor nerve fibers [97]. Nevertheless, as graft-derived NC cells are detected in the adrenal medulla following transplantation of cranial neural primordium to the adrenomedullary region [171], the ability to generate chromaffin cells is not limited to trunk NC.

1.7 Axial-specific gene regulatory networks

It has been proposed that the distinct properties of NC cells at various axial levels may be explained by axial differences in their GRNs [324]. Several transcription factors, including Id2 [199] and Ets1 [337, 347], are expressed in cranial, but not trunk, NC cells in chick embryos. However, it should be noted that chick Id2 is also expressed in cardiac NC cells [200] and Ets1 is expressed in zebrafish and hPSC-derived trunk NC cells [93, 106, 197]. Thus, a greater understanding of the regulatory functions of these factors, as well as the species-specific variation in the mechanisms that establish axial identity, is still needed. Nevertheless, Ets1 is both necessary and sufficient to confer cranial-specific delamination properties on NC cells in chick embryos [347]. In addition, the regulatory regions of two key NC specifier genes in chick—*Foxd3* [326] and *Sox10* [23]—have axial-specific enhancers that drive expression in either the cranial or the trunk NC [23, 326]. Notably, both cranial enhancers are directly activated by Ets1. In recent years, next generation sequencing approaches, primarily in

amniote model systems, have enabled researchers to evaluate the hypothesis that axial-specific GRNs pattern the NC.

Transcriptional profiling has further elucidated the gene regulatory differences between cranial and trunk NC cell populations. For example, Simões-Costa and Bronner (2016) [325] uncovered a cranial-specific transcriptional circuit in chick embryos. This GRN includes *Brn3c*, *Lhx5*, and *Dmbx1*, which are expressed in the anterior region of gastrula-stage embryos and persist throughout NC specification. Subsequently, *Tfap2b*, *Sox8*, and *Ets-1* are detected in NC progenitors in the cranial neural folds and in migrating NC cells. Introducing the latter three components of this network into the trunk is sufficient to reprogram trunk NC cells to a cranial identity and leads to the acquisition of chondrogenic potential [325]. A similar approach identified a transcriptional subcircuit comprised of *Tgif1*, *Ets1*, and *Sox8*, that imparts cardiac NC identity and is necessary for proper heart development. Ectopic expression of this subcircuit is sufficient to reprogram trunk NC cells to a cardiac fate and enables them to rescue defects in heart formation caused by cardiac NC ablation [100].

By coupling transcriptional and epigenomic profiling in cranial NC cells at population and single-cell levels, Williams et al. (2019) [375] reverse engineered the global NC GRN with remarkable resolution. Their analysis of chromatin dynamics revealed three distinct classes of regulatory elements: one accessible in the premigratory and/or migratory NC, one accessible in both NC and neuroepithelial cells, and one accessible in naive epiblast and premigratory NC cells but inaccessible at later stages [375].

Using a similar approach, Ling and Sauka-Spengler (2019) [185] dissected the GRN that governs the development of the vagal NC. Their study showed that this heterogeneous cell population can be separated into a $\text{Sox10}^{\text{high}}$/FoxD3-positive sub-population capable of forming neural, mesenchymal, and neuronal derivatives, and a $\text{Sox10}^{\text{low}}$/FoxD3-negative sub-population that is restricted to neuronal and mesenchymal fates. By incorporating chromatin accessibility and genetic interactions, this study identified the Tfap2, Sox, Hbox and

bHLH families of transcription factors as core regulators of the vagal crest GRN and validated their function by genetic knock-out [185].

Single-cell analyses of mouse embryos have revealed that NC cells at distinct axial positions exhibit largely similar transcriptional profiles over time, yet they also have important axial-specific biases [330]. For instance, cranial NC cells are biased towards a mesenchymal fate, whereas trunk NC cells are biased towards sensory and autonomic neuronal fates. These biases emerge during delamination, with mesenchymal fates resulting from sustained high levels of expression of *Twist1* in the cranial region [330]. Interestingly, cranial and trunk NC cells become transcriptionally distinct at different times in mouse and chick: while the mouse cranial program is established during delamination, the chick cranial GRN initiates during the early stages of NC specification [325].

Cranial NC cells in zebrafish also express *twist1*, which promotes ectomesenchymal fate at the expense of other genetic programs [63]. In mice, *Twist1* mutants show impaired skeletogenic differentiation and fail to form bones of the snout, upper face and skull vault [25, 331]. In both species, Twist1 deficiency leads to persistent expression of Sox10 and a loss of ectomesenchymal differentiation markers [25, 331, 63]. Soldatov and colleagues also showed that loss of *Twist1* in mouse cranial NC results in a reduction of mesenchymal derivatives and an increase in glial and neuronal fates. Conversely, ectopic expression of *Twist1* in the mouse trunk NC, starting from pre-EMT stages, results in the expression of a mesenchymal marker (*Prrx1*) at the expense of neuronal sensory, autonomic, and glial fates [330]. Together, these results indicate that Twist1 is sufficient to drive the acquisition of some ectomensenchymal fates.

Recent advances in the dissection of genetic circuits and interrogation of transcriptional profiles have been invaluable in uncovering the molecular basis of NC axial identity. These approaches have revealed that intrinsic differences in gene expression mediate at least some axial-specific properties of NC cells, including ectomesenchymal potential, and have begun

to establish the regulatory logic that underlies the cranial genetic circuit.

1.8 Differences in migratory behaviors between cranial and trunk NC cells

A recent report from the Linker lab reveals that cranial and trunk NC cells utilize distinct migratory strategies (Figure 1.6). Cell tracking experiments in both chick and zebrafish show that in the cranial region NC cells dynamically rearrange within the multicellular streams without regard to their initial time of emergence (Figure 1.6A). Further, when cells at the front of a cranial NC stream are ablated in zebrafish, migration remains unperturbed [287]. In the trunk, zebrafish NC cells consist of three distinct subpopulations: motile premigratory cells that remain in the dorsal region, leader cells that initiate migration along the medial pathway, and follower cells that trail the leader cell and connect it to the premigratory population (Figure 1.6B). Leader cells retain their position at the front of each stream throughout migration, whereas follower cells actively rearrange within a stream. Using laser ablation, Richardson and colleagues demonstrated that the leader cells are required for, and direct, trunk NC cell migration. Further, leader and follower identities are non-interchangeable and seemingly acquired before migration. After leader cell ablation, migration pauses until a new leader emerges from within the premigratory cell population, and migrates down the stalled stream to resume leading it onward [287]. Here, I describe the migration pathways of cranial and trunk NC cells in zebrafish embryos and summarize the molecular mechanisms that orchestrate this process.

1.8.1 Cranial NC migration

Zebrafish cranial NC cells begin their migration around 13 hpf. A subset of NC cells from the midbrain migrate anteriorly between and around the eyes to contribute to the neurocranium

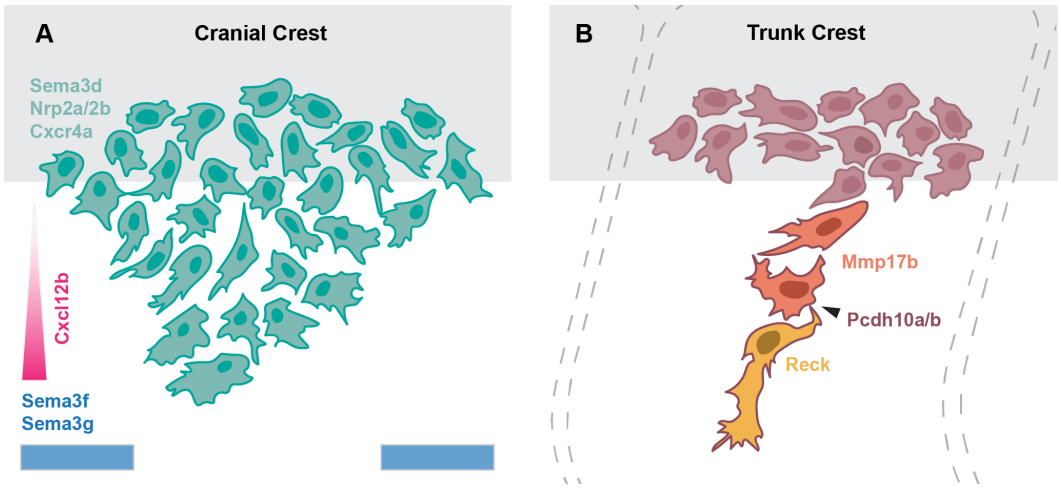

Figure 1.6: **Differences between cranial and trunk NC cell migration behaviors.** A, Schematic of molecular regulators of cranial neural crest migration. A stream of cranial NCCs migrates away from the neural tube (grey) avoiding repulsive Sema3f and Sema3g cues in the environment, shown in blue, and towards the chemokine Cxcl12b, shown in pink. These cells express Sema3d, as well as the receptors Nrp2a/2b and Cxcr4a. B, Schematic of trunk neural crest cell migration, away from the neural tube (grey), depicting the leader (yellow), follower (orange), and premigratory (red) populations. Leader cells express Reck, whereas follower cells express Mmp17b. All trunk neural crest cells express Pcdh10a/b, which mediates cell–cell contacts (arrowhead). Segmental migration of trunk neural crest cells is restricted by adaxial cells; see text for details.

[161] (see 1.5.2), whereas posterior midbrain and hindbrain-derived NC cells segregate into distinct streams that migrate ventrally into the pharyngeal arches (PAs) and produce the viscerocranium (Figure 1.1) [161, 306]. NC cells from the midbrain and rhombomeres (r) 1-3 contribute to the mandibular arch (PA1), while those from r3-5 populate the hyoid arch (PA2), and cells from r5-r7 fill the branchial arches (PA4-7) [306]. Studies from multiple model organisms have revealed that cranial NC cell migration is regulated by a variety of repulsive cues and chemoattractants (Figure 1.6A). Of note, semaphorins and their neuropilin/plexin receptors have been shown to play an important role in zebrafish cranial NC stream formation, while Sdf1 (Cxcl12b) is an important chemoattractant [99, 117, 349, 350]. However, many of the signals that have been demonstrated to control directional migration in NC cells of other species [350], including Eph/Ephrin signaling and VEGF, have not been evaluated in zebrafish.

Zebrafish *sema3d* is expressed as early as 11 hpf in premigratory NC cells and is eventually expressed in all three streams of NC cells that migrate towards the pharynx [22, 118]. Knockdown of Sema3d reduces migratory NC cell marker expression and disrupts NC cell-derived tissues [22]. Further, *sema3f* and *sema3g* are expressed in the NC-free regions of the head and coordinate the migration of cranial NC cells, which express the corresponding receptors *nrp2a* and *nrp2b* [385]. In addition to environmental repulsive cues, small, secreted chemoattractants are important for directing NC migration [350]. In zebrafish, *cxcr4a* is expressed in migrating cranial, but not trunk, NC cells, while *sdf1b* is expressed in the pharyngeal endoderm, the target tissue of directionally migrating cranial NC cells [260]. Knockdown and overexpression approaches have indicated that Sdf1b/Cxcr4a signaling promote proper migration of NC cells into the pharyngeal arches [260], possibly mediated via Fascin1-dependent filpodia [27]. Finally proper dispersal and migration of the anterior population of NC cells that contribute to the palate relies on an interplay of the attractant signal Pdgf, and its negative regulator, microRNA Mirn140 [80].

1.8.2 Trunk NC migration

Along the AP extent of the trunk, NC cells migrate on two distinct pathways: an initial, medial pathway, between the neural tube and the somites (Figures 1.2, 1.6B), and a later, lateral pathway, between the somites and the overlying ectoderm [283]. NC cells located at the most lateral aspect of the dorsal neural tube are the first to begin migration and enter the medial pathway starting after 15 hpf. The onset of their migration shows a distinct anterior to posterior sequence, although occasionally more posterior NC cells do migrate out of sequence, ahead of more anterior ones [283]. NC cells that migrate along the medial pathway give rise to all types of derivative, including neurons of the sensory and sympathetic ganglia, Schwann cells, and pigment cells (Raible and Eisen, 1994). Approximately 4 hours later, NC cells initially located in the dorsal-most region of the neural tube begin to enter the lateral pathway [283]. Notably, these cells only differentiate into pigment cells [282]. However, factors other than time of migration likely regulate pathway decision, as some cells continue to enter the medial pathway even after migration along the lateral pathway has begun [283]. Here, I will focus on the mechanisms that regulate migration along the medial pathway.

As in other species, zebrafish NC cell migration on the medial pathway is spatially restricted along the AP axis, such that a series of reiterated streams, one per somite, is established along each side of the trunk (Figure 1.1) [283]. However, unlike in chick and mouse embryos, where NC cells migrate through the sclerotome of the anterior half of each somite [28, 245], zebrafish trunk NC cells show no obvious AP restrictions until they reach the ventral edge of the neural tube, at which point they become restricted to migrating on the medial surface of the center of each somite (Figure 1.6B) [283]. In zebrafish, as in other teleosts, the majority of somite-derived cells are myotomal, with only a small ventrally localized sclerotome compartment forming [133, 231]. Ablation of the zebrafish sclerotome does not disrupt the segmental pattern or subsequent development of dorsal root ganglia

(DRG) [231]. Instead, the slow muscle precursors, termed adaxial cells, regulate the pattern of NC migration on the medial pathway [133]. This is perhaps not surprising as myotome cells are the first encountered by migrating NC cells and, in fact, the onset of NC migration coincides with contact between the two cell types [283].

Although the molecular basis of trunk NC cell stream restriction and migration has not been fully established, interactions between migrating NC cells and myotome cells, the extracellular matrix, and other NC cells are likely critical. The muscle-specific receptor kinase (MuSK) and its ligand Wnt11r play a key role in restricting NC cell migration to the center of each somite [12]. In the absence of MuSK, NC cells do not retract non-productive leading edges, leading to impaired stream formation [12]. Similarly, the glycosyltransferase *lh3* (now *plod3*) is expressed in a subset of ventral somitic cells and, with its substrate Collagen18A1, is necessary for segmental stream formation [12]. Additionally, the GPI-linked matrix metalloproteinase *mmp17b* and the metalloproteinase inhibitor *reck* are both required in a cell-autonomous fashion for NC cell migration [176, 278] (Figure 1.6B). Interestingly, Mmp17b and Reck are expressed in NC cells found in close apposition, and interact biochemically, raising the possibility that they function in concert [176]. Finally, loss of the proto-cadherin encoding gene *pcdh10a*, either singly or together with its paralog *pcdh10b*, disrupts migration along the medial pathway, in part due to a loss of cell-cell contact with other migrating NC cells [373].

1.9 Lessons from stem cells

The ability to differentiate human pluripotent stem cells (hPSCs) into NC cells *in vitro* has provided novel insights into the mechanisms by which the NC is patterned along the AP axis. Importantly, it has also proven an important tool for studying human NC biology and NC-associated developmental disorders. Early methods for deriving NC cells from hPSCs relied on stromal co-culture [143, 174, 274] or induction of neural rosettes [44, 173]. However,

these protocols yielded limited numbers of NC cells and often required FACS isolation using the cell surface markers HNK-1 and p75. More recently, several protocols have described feeder-free conditions for generating NC cells with high efficiency using small molecules and growth factors [113, 173, 179, 217, 216, 221].

Remarkably, these protocols yield hPSC-derived NC cells that possess cranial identity by default, indicated by their ability to give rise to chondrocytes and their lack of Hox expression (Figure 1.7) [96, 113, 174, 173, 179, 217, 221]. Treatment of hPSCs with retinoic acid (RA) during differentiation yields a subpopulation of NC cells with characteristics of cardiac and/or vagal NC, including expression of PG1-5 Hox genes (Figure 1.7) [93, 96, 221]. In particular, these conditions yield cultures with the potential to form enteric neurons, a cell type that defines the vagal NC [14, 87, 378]. Huang et al. (2016) [137] reported that, when combined with both TGF-β inhibition and Wnt signaling activation, treatment with RA generates NC cells that express PG6-9 Hox genes in addition to PG2-5 Hox genes (Figure 1.7). These NC cells activate the *Sox10E1* enhancer, which is expressed in both vagal and trunk NC, and they are capable of differentiating into TH+ sympathoadrenal cells [137]. However, the expression of PG6-9 in these cells is relatively low and unlikely to efficiently generate trunk NC cells. Finally, when NC cells are derived from stem cells in the presence of RA, they give rise to enteric neurons when grown together with human intestinal organoids, or colonize the foregut when transplanted into chick embryos [378]. These findings are consistent with the known role of endogenous RA, which is necessary for proper development and gut colonization of the enteric NC [248, 361]. Together, these studies indicate that treatment with RA during differentiation yields vagal NC cells.

In recent years, increasing evidence has suggested that the production of *bona fide* trunk NC cells from hPSCs requires passing through an intermediate state that resembles neuromesodermal progenitors (NMPs). NMPs are bipotent stem cells found in the primitive streak and tailbud that produce much of the trunk and tail, as described in section 1.10.

Initial studies suggested that hPSC-derived NMP-like cells, marked by robust co-expression of Sox2 and Brachyury/T, can be differentiated into trunk NC cells capable of differentiating into chromaffin cells *in vitro*, as well as *in vivo* upon transplantation into chick embryos [3, 71]. Subsequently, Frith and colleagues demonstrated that hPSC-derived NMPs [110] can differentiate into trunk NC cells and their derivatives [93, 94]. Interestingly, several known markers of neural plate border and early NC identity are also detected in these NMPs [93]. Under differentiation conditions, the hPSC-derived NMPs give rise to NC cells that express PG5-9 Hox genes—typical of the thoracic neurectoderm—and can also give rise to sympathoadrenal cells [93]. Other protocols for generating trunk NC cells—defined by expression of HoxC9, limited mesenchymal potential, and the ability to produce sympathoadrenal cells—again report the presence of an NMP-like intermediate state [107, 114]. Notably, these trunk NC cell cultures exhibit a wider developmental potential than avian trunk NC cells, as they are capable of forming smooth muscle and osteoblasts [107, 114]. Finally, NMP-derived preneural progenitors give rise to trunk NC cells with progressively more posterior identity over increasing passages [57], perhaps reflecting the colinear expression of Hox genes observed *in vivo*.

Additional experiments have revealed that Wnt and Fgf signaling, which are necessary for maintaining the NMP niche *in vivo* (reviewed in [376]) are also critical for specifying the axial identity of hPSC-derived NC cells *in vitro*. Wnt signaling levels are crucial for determining cranial versus trunk fate hPSC-derived NC cells [107, 114]. hPSCs exhibit a bimodal response to Wnt signaling, whereby low Wnt signaling leads to anterior, Hox-negative NC cells, and high Wnt signaling results in posterior, Hox-expressing NC cells [107]. Further, the magnitude of Wnt stimulus dictates the degree of NC posterior identity based on Hox gene expression, suggesting a rheostat response. Within the trunk compartment, Fgf signaling determines axial identities: treatment of hPSC cultures with Fgf2 during the first 2 days of NC induction leads to expression of the sacral HoxA10-13 genes (Figure 1.7),

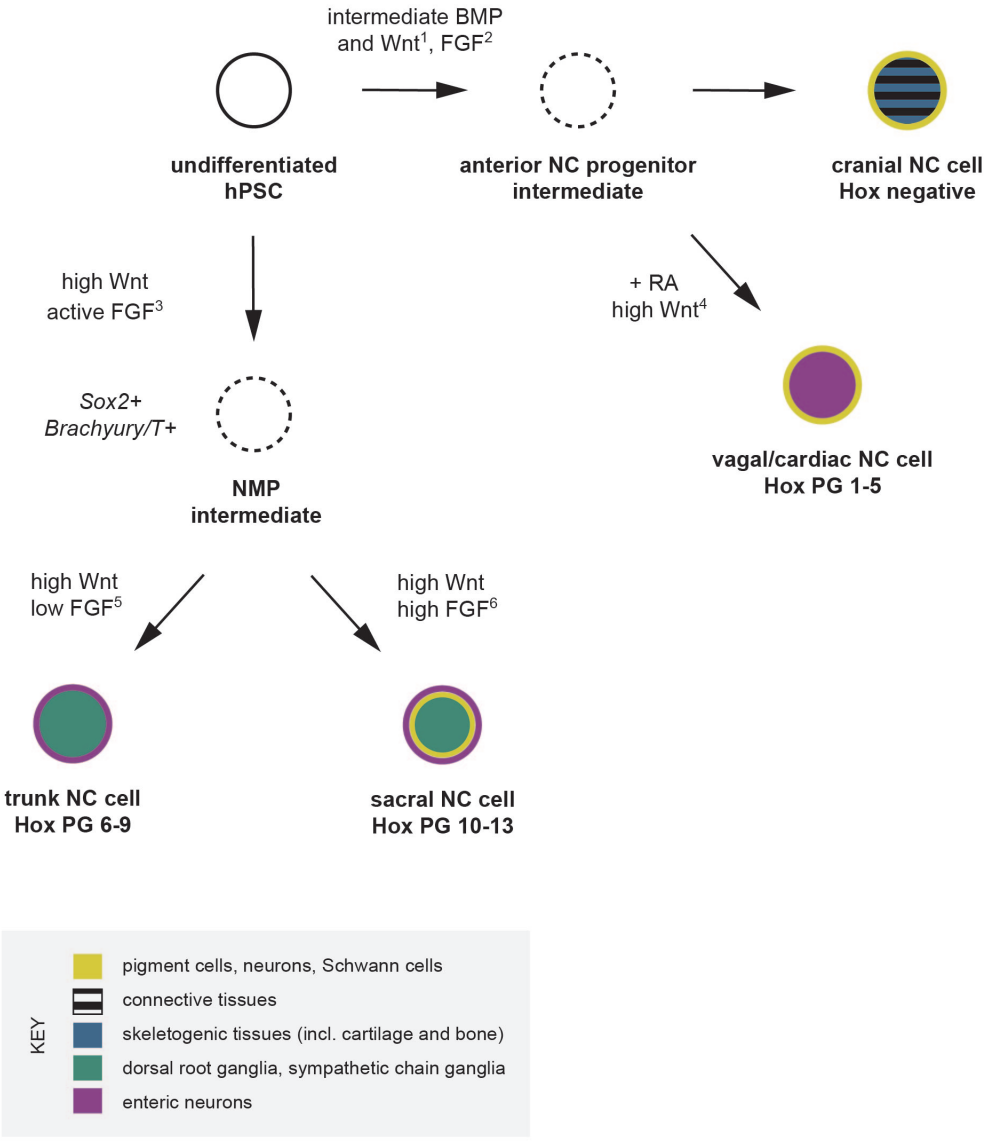

Figure 1.7: **Differentiation of hSPCs into distinct axial subpopulations of NC.** An undifferentiated human pluripotent stem cell (hPSC) passes through different intermediate states en route to a cranial or trunk neural crest cell fate. Each cell type and its characteristic gene expression is indicated. The signals needed to promote each cell type are indicated next to the arrows. Derivatives formed by each cell type are color coded. (1) Hackland et al., 2017; (2) Lee et al., 2007; (3) Frith et al., 2018; (4) Fattahi et al., 2016; (5) Gomez et al., 2019b; (6) Hackland et al., 2019.

whereas the FGF inhibitor PD17 abrogates all Hox expression [114].

The finding that NMPs produce trunk NC cells *in vitro* is consistent with the results of lineage tracing studies *in vivo*. Based on their analyses of chick and mouse embryos, Schoenwolf et al. (1984; 1985) [309, 308] first proposed that cells in the tail bud might give rise to NC cells in the tail. This hypothesis was later substantiated by grafting quail tissue into the tailbud of 25-somite stage chick hosts, which revealed that the cells in the chordoneural hinge region of the tailbud contribute not only to the spinal cord and somitic mesoderm—as expected of NMPs—but also to the NC and its derivatives [42]. More recently, fate-mapping of the mouse primitive streak and tailbud, either by grafting GFP-labeled cells or by permanent genetic cell labeling, has also shown that NMPs give rise to trunk and tail NC cells [142, 290, 359, 379], as well as NC-derived sensory neurons of the dorsal root ganglia in the sacral region [317]. However, the extent to which NMPs contribute to the NC remains unclear, as these analyses are based on only a small number of clones. Cre-based lineage tracing driven by *Tbx6* and *Nkx-2* regulatory sequences, which are expressed in NMPs and early mesodermal and neural progenitors, labeled NC cells in the anterior trunk [142, 290], although Javali et al. (2017) reported a higher contribution to NC cells at the sacral level. Clonal analysis using the *R26nlaacZ* system—in which an inactive variant of *LacZ* is driven by the ubiquitous Rosa26 promoter and rare spontaneous deletions restore β-galactosidase activity in single cells—yielded clones that labeled the NC along the entire extent of trunk and tail [359]. Collectively, these results provide compelling evidence that NMPs contribute to at least a subset of trunk NC cells *in vivo*.

Despite the evidence for a role for NMPs in generating trunk NC cells, it remains unclear whether NMP intermediates are necessary for trunk NC identity *in vivo*. In fact, Gomez et al. (2019) [107] also showed that cells exposed to high Wnt can still adopt trunk NC fate even when expression of NMP markers is compromised following inhibition of Fgf signaling. Additionally, it is unclear how the NMP state might imbue trunk-specific features

of NC cell development, such as the restriction of ectomesenchymal potential. Thus, it is important to better elucidate the regulatory link between the NMP state and trunk NC identity, which is a focus of Chapter 2 of this thesis. Moreover, while the contribution of NMPs to axial elongation and NC cell populations has been well documented in amniotes, whether equivalent cells play a role in the development of non-amniotes, such as zebrafish [11, 148, 198] and *Xenopus* [108], remains controversial. Nevertheless, some have proposed that the molecular mechanisms governing posterior extension of the embryo—including the gene regulatory state that defines NMPs—are conserved across vertebrates [155, 333, 376]. As current experimental evidence for this assertion remains inconclusive, one of the goals of this thesis is to investigate whether the genetic circuits and signaling pathways that define the NMP-like state are present in zebrafish embryos, and explore how these are linked with the specification of NC fate in the posterior body.

1.10 Neuromesodermal Progenitors

In mouse and chick, tissues posterior to the head are in large part generated by multipotent stem cells, termed neuromesodermal progenitors (NMPs). This idea was first supported by lineage tracing of Hensen's node in the chick embryo [315]. Specifically, by labeling small groups of cells with DiI or single cells by lysine-rhodamine-dextran (LRD) injection, Selleck and Stern (1991) first demonstrated that Hensen's node is spatially and temporally organized [315]. Strikingly, these experiments also uncovered that single cells give rise to progeny located in more than one tissue type. Finally, the labeling of cells that contribute to the notochord or somites also revealed that their progeny were arranged in a periodic fashion [315].

The first indication that the post-cranial neural tube, notochord, and somites might originate from a resident stem cell population came from the clonal analysis of mouse somitic and spinal cord progenitors using the *laacZ* system [202, 247]. Expression of *laacZ* under

the regulation of a myotome specific promoter, resulted in "long" clones that extended to the posterior end of the embryo from their anterior border [247]. Based on the retrospective analysis of these clones, Nicolas and colleagues (1996) concluded that the myotome is derived from a spatially organized pool of self-renewing stem cells that follows axis formation during development [247]. Similarly, the retrospective clonal analysis of spinal cord progenitors also supports the involvement of a self-renewing population of stem cells [202].

Since, careful fate-mapping of the mouse primitive streak and the adjacent epiblast—the caudal lateral epiblast (CLE) and the node-streak border— and later in the chordoneural hinge (CNH) has shown that these domains contain resident stem cells that give rise to both neural and mesodermal derivatives [30, 36, 36, 359, 379]. Furthermore, grafts from the CLE and CNH can be serially transplanted into earlier-stage hosts [36, 37], and are capable of resetting their Hox identity to match their new environment [209]. Together, these studies defined the functional hallmarks of NMPs, namely their multipotency and stem cell nature.

In addition to their functional characterization, remarkable progress has been made towards discovering the signaling and gene regulatory basis for the establishment, maintenance, and differentiation of NMPs. In particular, NMPs are defined by the co-expression of the neural marker *Sox2* and the mesodermal market *T/Brachyury* [37, 333, 376]. Interactions between Wnt, Fgf, and Retinoic Acid (RA) signaling orchestrate the self-renewal and differentiation of NMPs [333, 376]. Mutants in the Wnt [104, 338], Fgf [50], and RA pathways [2] are characterized by axial truncations. In recent years, next generation sequencing approaches and the capacity to differentiate hPSCs into NMPs [110] have allowed researchers to organize these extracellular signals and cell-intrinsic gene regulatory interactions into a GRN that underlies development of NMPs [109]. Importantly, Cdx factors are a core-component of the NMP GRN, as described in 2.

While NMPs have been well characterized in amniotes, their existence and contributions to the post-cranial tissues in non-amniotes, including zebrafish, findings remain controversial

due to limited experimental data. Nevertheless, it has been argued that the mechanisms governing posterior body growth are conserved across vertebrates [155, 376], particularly if NMPs are viewed as a transition state from which neural or mesodermal stable states emerge [333].

Martin and Kimelman (2012) identified a population of cells that co-express *sox2* and the zebrafish ortholog of *T/Brachyury*, *tbxta* [198]. These cells have the capacity to give rise to either mesoderm or neural cell types under experimental conditions, yet typically produce mesoderm in response to Wnt signaling [198]. However, it should be stressed that these NMP-like cells of the zebrafish tailbud show significant differences from amniote NMPs. Most importantly, lineage tracing studies of individual zebrafish tailbud cells, either by dye labeling [148] or photolabeling [11], have only found mono-fated progenitors in the tailbud. In fact, lineage analysis based on *in vivo* imaging suggests that bipotent, "NMP-like" cells rapidly segregate into neural and mesodermal cell fates early during gastrulation [11]. This is in stark contrast to the results of lineage-tracing studies in amniotes, which have uncovered clones that contribute to both neural and mesodermal derivatives over extended periods during posterior body outgrowth [36, 37, 209, 379].

Furthermore, cells of the zebrafish tailbud exhibit low proliferation rates [11, 148]. In fact, the post-gastrulation 'NMP-like' cells described by Attardi and colleagues (2019) are largely quiescent, mono-potent, and contribute only to the posterior-most region of the body axis[11]. Again, this is a marked difference from amniote NMPs, which are proliferating stem cells that contribute to both mesoderm and neural tissue at all postcranial axial levels [376]. Based on this evidence, I contend that the *sox2/tbxta*-expressing cells of the zebrafish tailbud do not exhibit the functional hallmarks of NMPs.

The points detailed above notwithstanding, if NMPs are viewed as a gene-regulatory state from which neural or mesodermal stable states emerge [333], it is possible that an NMP-like state may be present in the zebrafish tailbud. In support of this view, a subset of

cells that co-express *sox2* and *tbxta*, a defining molecular characteristic of the NMP state in amniotes have been detected in the zebrafish tailbud [198, 11]. Moreover, the outgrowth of the zebrafish posterior body is dependent on the same extracellular signals as in amniotes, namely Wnt and Fgf. As in amniotes, inhibition of Wnt [121, 177, 322] or Fgf [77, 112] signaling results in posterior truncations in zebrafish. Finally, Cdx factors are also necessary for outgrowth of the posterior body in zebrafish (see Chapter 1.11). Therefore, in Chapter 2 of this thesis, I investigated the role of Cdx4 in regulating Wnt and Fgf signaling and the genetic circuit that drives posterior body outgrowth in zebrafish embryos. My analysis suggests that Cdx4 may link the regulatory networks the underlie posterior body formation and NC cell specification.

1.11 Cdx Factors in AP patterning

A particularly exciting candidate for conferring posterior identity onto the NC is the family of caudal-related homeobox (Cdx) proteins. In amphioxus, *AmphiCdx* is contained within the ParaHox cluster, along with *AmphiXlox* and *AmphiGsx*. Significantly, genes of the ParaHox cluster are expressed in a spatial order along the body axis that matches their genomic organization [29]. Therefore, it is likely that both the Hox and ParaHox gene clusters arose from a common precursor, termed the ProtoHox cluster [29, 88]. Amniote genomes contain three members of the caudal homeobox family, termed *Cdx1*, *Cdx2*, and *Cdx4* based on syntenic arrangement [135, 188, 383], which appear to play a conserved role in patterning the anteroposterior axis, as well as vertebral patterning, trophoblast function, and intestinal development [135].

The teleost zebrafish also possesses three *cdx* genes, despite an additional round of whole genome duplication in the lineage leading to teleosts. The three gene arrangement reflects gene loss, subsequent to the duplication event, which eliminated both copies of *cdx2* and one copy of *cdx4* [235, 280, 323]. Zebrafish *cdx1b* is primarily involved in endoderm development

and does not exhibit a specific expression pattern until 24 hpf [47, 89]. Both *cdx1a* and *cdx4* are expressed from the early gastrula stage in the ventral blastoderm margin [146, 65, 66, 322]. During segmentation stages, both *cdx1a* and *cdx4* are present in the developing tailbud [146, 65, 66, 322], yet only *cdx4* is additionally expressed in prospective spinal cord territory with an anterior limit at the boundary with the posterior hindbrain [146, 65, 66, 322]. The zebrafish *cdx1a* and *cdx4* genes have both redundant and independent functions. Loss of Cdx4 function alone impairs posterior body outgrowth [65], whereas loss of Cdx1a function alone does not result in overt morphological defects [66]. Yet, abrogation of both Cdx1 and Cdx4 function leads to a more severe truncation phenotype [66, 327].

In zebrafish embryos, Cdx factors pattern the nascent neural plate along the AP axis. In particular, Skromne and colleagues (2007) [327] have demonstrated that Cdx factors specify the spinal cord by repression of the hindbrain developmental program, as loss of Cdx1a and Cdx4 leads to an expansion in the hindbrain at the expense of spinal cord. Cdx factors also play a similar role in mouse embryos, where they mediate the chromatin remodeling events necessary for the acquisition of spinal cord fate and the concomitant repression of hindbrain fate [218]. Additionally, during the course of this dissertation, articles were published suggesting that Cdx factors function as early NC specifiers in mouse embryos—where they directly activate the expression of NC specifier genes, including *Foxd3*, *Pax3*, and *Msx1* [296, 295, 297]— and during hPSC differentiation into trunk NC cells [93]. Therefore, I set out to investigate the role of Cdx factors in the development of NC cells in the posterior body of the zebrafish.

1.12 Thesis Overview

In this chapter, I have described the mechanisms that underlie NC cell development, with a focus on findings made in zebrafish. I have also summarized our current understanding of the mechanisms by which the NC is patterned along the AP axis into distinct subpopulations

that exhibit unique differentiation potential, genetic circuits, and cellular behaviors during migration. Most studies to date have focused on cranial NC cells, and, as a consequence, detailed understanding of the mechanisms that govern the development of NC cells in the posterior body remains an important unaddressed question in the field. Thus, my thesis aims to address this gap in knowledge.

In this thesis, I have investigated the role of zebrafish *cdx4* in the development of NC cells in the posterior body. In Chapter 2, I describe how Cdx4 functions as a regulatory link between the genetic circuits that orchestrate NC specification and posterior body outgrowth. This work relied on analysis of previously-reported ChIP-seq and microarray datasets, as well as my experimental validation of candidate regulatory targets of Cdx4 using a novel mutant. In Chapter 3, I analyze the role of *cdx4* in patterning the NC along the AP axis. Specifically, I used high-resolution microscopy to demonstrate that *cdx4* is necessary for the trunk-specific cellular behaviors that underlie segmental migration of NC, likely via the regulation of leader cell identity. Further, by generating and analyzing chimeric embryos I was able to show that *cdx4* function in somitic cells is not necessary for proper segmental migration of trunk NC cells.

In the two appendices (A and B) I summarize additional lines of research completed in the course of my thesis research. In Appendix A, I evaluate whether *cdx4* is necessary for repressing chondrogenic potential in trunk NC cells. Based on my results, I conclude that *cdx4* function in trunk NC cells is neither necessary not sufficient for regulating this property. In Appendix B, I describe transcriptomic profiling of cranial and trunk NC cells, which has identified candidate genes for patterning the NC along the AP axis. In Chapter 4, I provide a description of the materials and methods used throughout my thesis research.

Taken together, the results I have presented in this thesis support my conclusion that *cdx4* functions as a key early NC specifier gene in the posterior body during zebrafish development. In Chapter 5 I place my results within the context of the published literature, especially in

regard to the function of other early NC specifiers. I also highlight the significance of my findings within the broader field of NC cell development and present ideas for future investigations.

CHAPTER 2

CDX4 FUNCTIONS AS A REGULATORY NEXUS BETWEEN TRUNK NEURAL CREST DEVELOPMENT AND POSTERIOR OUTGROWTH

2.1 Abstract

Neural Crest (NC) cell specification progresses in an anterior to posterior fashion and results in distinct, axial-restricted subpopulations. While the anterior-most, cranial, population of NC cells is specified as gastrulation concludes and neurulation begins, more posterior populations become specified as the body elongates. Thus, establishment and patterning of posterior NC must be coordinated with the outgrowth of the posterior body. However, how these two processes are coordinated remains unknown. Here I provide genetic and genomic evidence that the zebrafish caudal-related homeobox transcription factor Cdx4 participates in the gene regulatory circuits that orchestrate both NC cell specification and outgrowth of the posterior body. I demonstrate that Cdx4 directly binds NC cell-specific enhancers of *foxd3*, a key gene in NC cell development. Moreover, the Cdx4 and Foxd3 factors share binding sites near many of the genes that mediate the early steps of NC cell specification. In addition, Cdx4 directly regulates the Wnt and Fgf signaling pathways, and binds the regulatory sequences of genes that drive posterior body formation and morphogenesis. Given these functions, I contend that Cdx4 functions as a regulatory nexus, potentially allowing for the coordination of two interlinked processes. Furthermore, these findings suggest broad conservation in the molecular control of posterior outgrowth across the vertebrates.

Attributions

The work summarized in this chapter was performed in collaboration with several members of the Prince and Ho labs. The bioinformatics analysis was performed by undergraduate students Ruby Schnirman and Clare Booth under my mentorship. Specifically, Clare Booth performed analysis included in Figures 2.1 and 2.2, and Ruby Schnirman performed analysis included in Figure 2.5, as well as performing HCR experiments included in Figures 2.1 and 2.5. Alana Beadell generated the $cdx4^{ch107}$ mutant line, Noor Singh performed the HCR experiments included in Figures 2.1, 2.4, and 2.6 and Anastasia Beiriger illustrated the schematics included in Figures 2.2 and 2.6.

2.2 Introduction

The neural crest (NC) is a transient, multipotent cell population that gives rise to an array of cell types, including chondrocytes, sensory neurons, glia, and pigment cells [169]. NC development proceeds in an anterior to posterior (AP) fashion and consists of induction and specification at the neural plate border (NPB), delamination from the neural epithelium, migration, and differentiation [170, 289, 324]. Careful analyses of early NC development in chick have revealed that NC induction and specification begin during gastrulation [17, 86] and continue concurrently with neurulation. Our current understanding of the molecular mechanisms that orchestrate NC induction and specification stems largely from studies carried out at gastrulation stages or shortly thereafter. However, much of the post-cranial body is generated over an extended period of time following the completion of gastrulation [376]. Post-cranial NC development and posterior body outgrowth must therefore be coordinated. Yet, how NC fate is specified during posterior body outgrowth remains unknown.

An appealing candidate to link axial outgrowth with NC specification can be found in the Caudal-related (Cdx) family of transcription factors. Cdx factors are expressed in the posterior of the developing body and play a conserved role in axial elongation and patterning

of the embryo along the AP axis [135, 188, 384]. In addition, Cdx factors directly activate key genes necessary for NC specification in mouse embryos, including *Pax3*, *Msx1*, and *Foxd3* [297, 296, 295]. Further, it has been hypothesized that Cdx factors are necessary for the establishment of neural plate border and NC identity in the posterior [93]. We therefore evaluated the model that Cdx factors function as a nexus between the genetic circuits that govern NC specification and axial outgrowth.

Cdx factors play a conserved role in driving outgrowth of the posterior body, in part via their function in an autoregulatory feedback loop with the Wnt and Fgf signaling pathways. Heterozygous mouse *Cdx2* mutants exhibit posterior truncations [46], a phenotype which becomes more severe in combination with loss of either *Cdx1* or *Cdx4* [362, 363, 384]. Further, ablation of all three murine *Cdx* genes results in complete loss of post-occipital tissues [365]. Similarly, abrogation of zebrafish Cdx function, either in mutants or by morpholino knockdown, leads to posterior truncations [65, 66, 327]. In both mouse and chick, Cdx factors are positively regulated by Wnt and Fgf signaling [19, 254, 270, 369]. The Cdx factors in turn regulate the expression of Wnt and Fgf ligands, as well as the RA-degrading enzyme Cyp26a [304, 384, 383]. Consequently, the truncation phenotypes observed in mouse Cdx mutants can be rescued by activation of the Wnt [383] and Fgf [365] signaling pathways. A similar auto-regulatory loop between Wnt, Fgf, RA signaling and Cdx factors has been described in zebrafish [281, 322, 321, 327].

This chapter combines analysis of the zebrafish *cdx4*^{ch107} mutant with genomic approaches to investigate the role of Cdx4 in both NC cell specification and growth of the post-cranial body. I demonstrate that zebrafish Cdx4 binds putative enhancers of the key NC specifier gene *foxd3* and regulates its expression in the posterior body. Additionally, analysis of shared binding targets of Cdx4 and Foxd3 suggests these two factors may co-regulate key genes of the NC gene regulatory network (GRN). I also show that zebrafish Cdx4 directly regulates genes of the Wnt and Fgf signaling pathways and influences Wnt

signaling in the developing tailbud. Further, zebrafish Cdx4, together with Tbxta, an ortholog of T/Brachyury [310], binds regulatory sequences near key genes in the tailbud GRN and is necessary for tailbud morphogenesis. Together, our findings suggest that zebrafish Cdx4 integrates the early steps of NC formation with the growth of the posterior body.

2.3 Results and Discussion

2.3.1 Cdx4 regulates expression of the NC specifier gene foxd3 in the developing tailbud

Foxd3 is a central component of the NC cell GRN [324] and functions as a key specifier of NC cell fate [163, 187, 229, 334, 136, 345]. As zebrafish *foxd3* is expressed in the developing tailbud as well as in premigratory NC cells [190, 256], its regulatory logic may be key to understanding how NC formation and posterior body outgrowth are coordinated.

Cdx factors have been shown to activate the expression of *Foxd3* in the NC of developing mouse embryos [295]. We began our analysis by investigating whether Cdx4 similarly regulates the expression of *foxd3* in developing zebrafish. We first assayed whether *cdx4* and *foxd3* are co-expressed in zebrafish embryos at 12 hours post fertilization (hpf), during early segmentation stages, using hybridization chain reaction (HCR). In accord with previous descriptions [256], we detected *foxd3* transcripts in early NC cells located within the dorsal neural tube of the spinal cord (not shown). Importantly, *cdx4* transcripts were also detected in the *foxd3*-expressing cells of the developing tailbud (Figure 2.1 A-D). We conclude that *cdx4* is active in the right place and time to regulate *foxd3*.

We next evaluated whether *foxd3* is a target of Cdx4 regulation. We first took a bioinformatics approach, by analyzing a previously reported ChIP-seq dataset generated using Myc-tagged Cdx4 in 10 hpf zebrafish embryos [262], a developmental stage that marks the end of gastrulation proper [156]. To uncover putative cis-regulatory regions, we assigned the

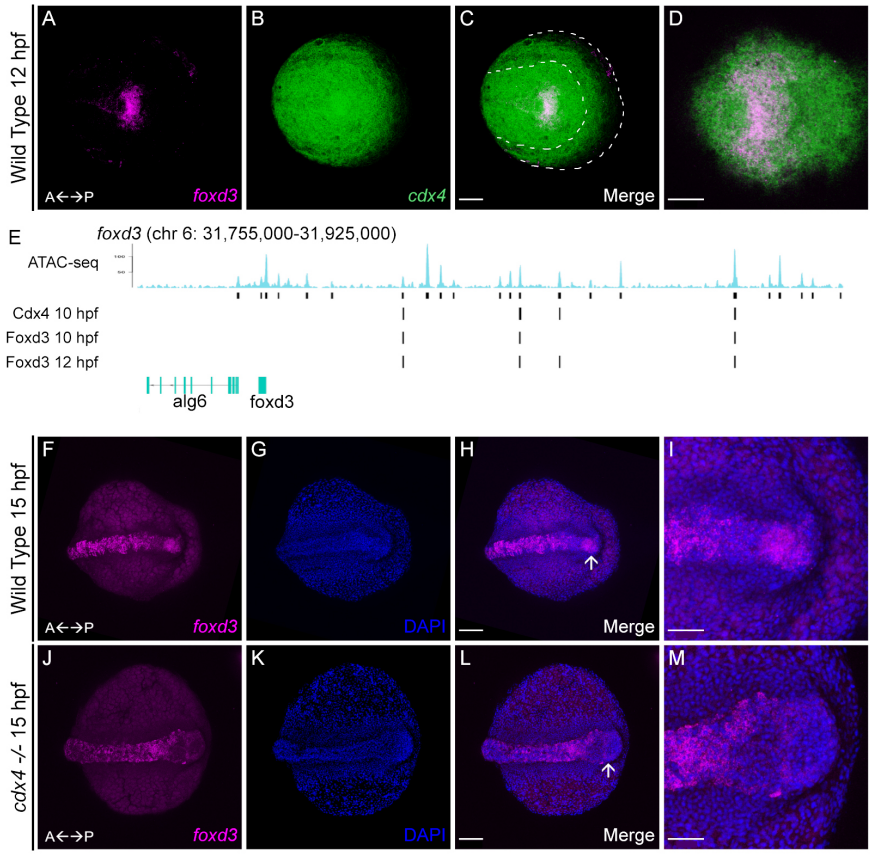

Figure 2.1: **Cdx4 regulates posterior expression of *foxd3*.** A. Dorsal image of *foxd3* RNA transcripts in the tailbud of 12 hpf embryos detected by HCR. B. Dorsal image of *cdx4* RNA transcripts in the tailbud of 12 hpf embryos detected by HCR. C. Merged image of A and B reveals co-expression of *cdx4* and *foxd3* transcripts at the tailbud. Dashed lines demarcate the outline of the tailbud (inner line) and the yolk; scale bar = 100 µm. D. Magnified 10 µm optical section shows co-detection of *foxd3* and *cdx4* transcripts in individual cells of the tailbud; scale bar = 50 µm. E. Genome browser data showing Cdx4 and Foxd3 binding near the *foxd3* locus. From top to bottom: mapped ATAC-seq in *foxd3*-expressing cells, with called peaks indicated below, Cdx4-Myc ChIP-seq peaks at 10 hpf, Foxd3-biotin-ChIP-seq peaks at 10hpf, and 12 hpf. F-G. Dorsal view of 15 hpf WT or $cdx4^{ch107+/-}$ heterozygous embryo shows *foxd3* RNA transcripts detected by HCR in the dorsal spinal cord and tailbud (F) counterstained with DAPI (G). H. Merged image of F and G; arrow indicates tailbud. Scale bar = 100 µm. I. Magnified view of tailbud region from H; scale bar = 50 µm . J-K. Dorsal view of 15 hpf $cdx4^{ch107-/-}$ mutant embryo shows *foxd3* RNA transcripts detected by HCR in the dorsal spinal cord and lack of expression in the tailbud (J) counterstained with DAPI (K). L. Merged image of J and K; arrow indicates tailbud. Scale bar = 100 µm. Magnified view of tailbud region from L; scale bar = 50 µm.

4,965 Cdx4-binding regions identified by Paik et al. (2013) to 2,407 zebrafish genes using GREAT [210]. To identify biologically-relevant binding sites, we incorporated previously reported ATAC-seq data (which identifies regions of open chromatin) from *foxd3*-expressing cells in 12 hpf embryos [190]. Our analysis revealed that Cdx4 binds four regions of open chromatin near the *foxd3* locus (Figure 2.1 E). Of note, three of these enhancers drive reporter expression in NC cells [190]. Moreover, all four of these regions have previously been reported as targets of Foxd3 binding [190], and are possible sites through which Foxd3 auto-regulates its expression [136, 187, 190].

Having established that Cdx4 binds the putative NC enhancers of *foxd3*, we tested whether Cdx4 function is necessary for normal *foxd3* expression. To facilitate this analysis, we used CRISPR/Cas9 to generate a new mutant in the *cdx4* gene, $cdx4^{ch107}$. This allele is characterized by an insertion/deletion after codon 28 that results in a frameshift and creates a premature stop at codon 102. Importantly, this yields a truncated protein that lacks the homeodomain and is thus a predicted null. As expected, the $cdx4^{ch107-/-}$ mutant closely resembles the previously described $cdx4^{kugelig}$ mutant [65]. By 15 hpf the $cdx4^{ch107-/-}$ phenotype can be unambiguously identified based on visual inspection of gross morphology. We therefore assayed *foxd3* expression at this stage using HCR on the progeny of crosses between $cdx4^{ch107+/-}$ heterozygous fish. Notably, the heterozygous siblings were indistinguishable from wild type (WT) specimens. In WT or heterozygous sibling embryos *foxd3* mRNA was detected in both the tailbud and in premigratory NC cells at the dorsal neural tube (Figure 2.1 F-I). By contrast, in $cdx4^{ch107-/-}$ mutant embryos *foxd3* expression was reduced in the dorsal neural tube and was undetectable in the developing tailbud (Figure 2.1 J-M) (n= 16). We conclude that zebrafish Cdx4 is necessary for the tailbud expression of *foxd3*.

Together, these data imply that Cdx4 directly regulates the posterior-most expression of *foxd3*. This finding is especially notable given that mouse Cdx2 has been hypothesized to

function as a 'pioneer factor' [7, 242], a class of transcription factors capable of binding to targets in silenced regions of chromatin and establishing competence for transcription [386]. Our results suggest that zebrafish Cdx4 may similarly act as a pioneer factor to initiate the expression of *foxd3* in the tailbud and subsequently in the dorsal spinal cord, a tissue that derives from the tailbud during axial outgrowth [148]. However, the presence of *foxd3* transcripts in the premigratory NC cells at the dorsal neural tube of *cdx4*$^{ch107-/-}$ mutants, albeit at reduced levels, implies that early tailbud expression of *foxd3* can be bypassed, and *foxd3* expression can eventually be activated by other means.

2.3.2 Cdx4 and Foxd3 both bind neural plate border genes

In zebrafish, Foxd3 has been shown to act as a pioneer factor that activates the expression of genes governing NC specification [190], which are often divided into the neural plate border (NPB) and premigratory NC modules [324]. In mouse, Cdx factors activate the expression of the NPB genes *Pax3* and *Msx1* [297, 296, 295]. Therefore, we hypothesized that zebrafish Cdx4 might cooperate with Foxd3 in regulating the expression of genes that mediate the early steps of NC specification.

To evaluate this hypothesis, we first examined the binding activity of zebrafish Foxd3 and Cdx4 at genes of the NC GRN as described by Simões-Costa and Bronner (2015) [324]. In particular, we focused on the genes that comprise the following modules: induction, NPB, premigratory NC, and migratory NC. We again analyzed the Cdx4 ChIP-seq dataset from 10 hpf embryos [262], as well as the ATAC-seq dataset from *foxd3*-expressing cells at 12 hpf [190]. In addition, we incorporated previously reported data from ChIP-seq of Foxd3 in 10 hpf and 12 hpf zebrafish embryos [190]. Using GREAT, we assigned 531 Foxd3-bound regions at 10 hpf and 2,955 regions at 12 hpf to 428 genes and 789 genes, respectively. Of the 50 genes that make up these four modules of the NC GRN, 13 were bound by both Cdx4 and Foxd3 at 10 hpf and 25 were bound by both Cdx4 at 10 hpf and Foxd3 at 12 hpf (Figure

2.2 A). It should be noted that there are two important limitations of this analysis. First, it uses two distinct datasets from ChIP-seq performed at slightly different developmental stages. Second, these data are derived from populations of cells rather than single cells. Therefore, I cannot definitively state that both factors bind simultaneously at these putative enhancers in any individual cell. Nevertheless, the data do reveal that Cdx4 and Foxd3 share target genes within the NC GRN, and suggest that they may co-bind within the loci of multiple genes.

Notably, a large proportion of genes (13 out of 17) that comprise the neural plate border module [324] were bound by both Cdx4 and Foxd3 at one or both of the stages analyzed. We therefore examined the binding activity of Cdx4 and Foxd3 near candidate NPB genes—*msx1a*, *pax3a*, *prdm1a*, and *tfap2a* (Figure 2.2 C-F)—and observed that the two transcription factors often bind the same putative cis-regulatory regions (Figure 2.2 B). Shared binding sites were uncovered at both proximal and more distal regions of open chromatin, and sometimes within intronic regions. We also observed binding of both Cdx4 and Foxd3 near genes of the pre-migratory NC module, including *sox9b*, *snai1b*, and *id2a* (Figure 2.2 G-I). However, in these genes the two factors do not occupy the same putative cis-regulatory regions (Figure 2.2 B). Finally, later-acting NC specifiers, such as *sox10* (Figure 2.2 J) and genes important for NC migration (data not shown), were not typically bound by both Cdx4 and Foxd3.

In summary, our results suggest that Cdx4 and Foxd3 transcription factors may co-regulate genes necessary for early NC specification, potentially interacting directly while binding at the same enhancers near key regulatory genes. This finding is in agreement with the previously suggested role for Cdx factors as NC specifiers in mouse embryos and hPSCs [93, 295]. Because Cdx factors play a well-documented role in driving outgrowth of the posterior body, we next investigated the molecular mechanisms by which Cdx4 may coordinate these two interlinked processes.

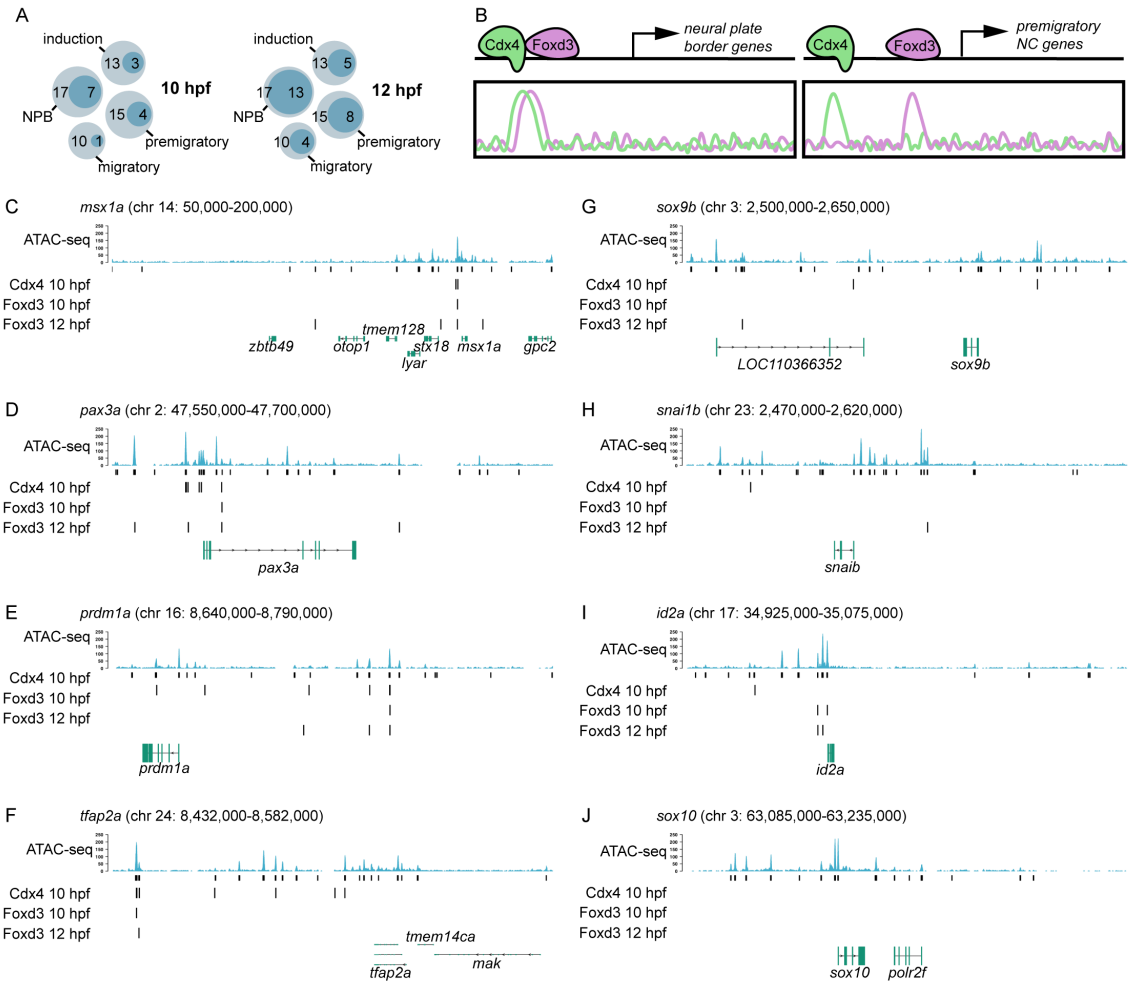

Figure 2.2: **Cdx4 and Foxd3 bind near early NC cell specification genes.** A. Schematic representation showing number of genes of each NC GRN module bound by both Cdx4 at 10 hpf and Foxd3 (dark circle) at 10 and 12 hpf out of the total number of genes in the module (light circle). B. Schematic representation of Cdx4 and Foxd3 binding near genes of the NMP (left) and NC specifier genes (right). C-F. Analysis of Cdx4 and Foxd3 binding near the NPB genes *msx1a* (C), *pax3a* (D), *prdm1a* (E), and *tfap2a* (F), as well as the premigratory and migratory NC genes *sox9b* (G), *snai1b* (H), *id2a* (I), and *sox10* (J). In all panels, the plots are laid out as follows (from top to bottom): ATAC-seq profiles from *foxd3*-expressing cells, with called peaks below, Cdx4-Myc ChIP-seq peaks at 10 hpf, Foxd3-biotin-ChIP-seq peaks at 10hpf, and 12 hpf, and Ref-seq annotated genes.

2.3.3 Cdx4 regulates Wnt and Fgf Signaling Pathways

Across vertebrates, interactions between Wnt, Fgf, and Retinoic Acid (RA) signaling orchestrate the development of the post-cranial body [155, 333, 376]. Consistent with these findings, mouse mutants in the Wnt [104, 338, 380], Fgf [50, 70], and RA pathways [2] are all characterized by axial truncations. Similarly, in zebrafish, inhibition of Wnt [121, 177, 322] or Fgf [77, 112] signaling results in posterior truncations. In mouse embryos, Cdx factors form an auto-regulatory loop with Wnt and Fgf signaling [304, 365, 383, 384], and mouse Cdx2 directly activates these two pathways in the developing tailbud [7].

In zebrafish embryos, similar regulatory relationships between Cdx factors and both Wnt and Fgf signaling have been described [322, 321]. Specifically, Wnt and Fgf signaling regulate the expression domain of *cdx1a* and *cdx4*, while, reciprocally, Cdx factors have been reported to shape the Fgf gradient in the posterior body [322, 321]. We once again used a bioinformatics approach to investigate the molecular mechanisms that govern this feedback loop. We first analyzed available microarray data from 11 hpf WT and Cdx4-knockdown (morpholino injected; morphant) zebrafish embryos [262]. This revealed 1,109 down-regulated and 1,342 up-regulated genes in Cdx4 morphants relative to WT embryos (p value ¡0.05). Moreover, of the down- or up-regulated genes, 188 and 142 genes, respectively, were directly bound by Cdx4. Gene ontology analysis of the differentially expressed genes revealed that 35 genes were part of the Wnt signaling pathway (Figure 2.3 A) and 17 genes belonged to the Fgf signaling pathway (Figure 2.33E). Importantly, several genes in the Wnt and Fgf pathways were both bound by Cdx4 and differentially expressed in Cdx4 morphants (Figure 2.3 B-D, F-H), suggesting that these are direct regulatory targets. In addition, Cdx4-binding sites fall within regions of open chromatin identified by ATAC-seq of *foxd3*-expressing cells from 12 hpf embryos [190] (Figure 2.3 B-D, F-H). These findings suggest that Cdx4 binds genes within the Wnt and Fgf signaling pathways at putative cis-regulatory regions and directly regulates their expression. This direct regulation likely underlies the

autoregulatory interactions between Cdx factors and Wnt and Fgf signaling in the developing posterior body.

Previous studies have reported that abrogation of Cdx function by morpholino-mediated knockdown of Cdx4, Cdx1a, or both, altered the Fgf signaling gradient in the posterior body [321]. We have begun to investigate whether Cdx factors also regulate Wnt signaling in the developing tailbud. We used HCR to assay expression of *wnt3a*, which encodes a canonical Wnt ligand, in the progeny of crosses between *cdx4*$^{ch107+/-}$ heterozygous fish. In WT and heterozygous 15 hpf siblings *wnt3a* was detected in the developing tailbud and in the dorsal neural tube at all axial levels (n = 16) (Figure 2.4 A-C). In *cdx4*$^{ch107-/-}$ mutant embryos, *wnt3a* was also detected in the tailbud and dorsal neural tube (n = 16) (Figure 2.4 D-F). Further, relative to WT embryos, the domain of *wnt3a*-expression in the tailbud of *cdx4*$^{ch107-/-}$ mutants was significantly shortened along the AP axis (Figure 2.4 A-F'). Further, our analysis also revealed that the posterior-most tailbud tissues are expanded laterally in *cdx4*$^{ch107-/-}$ mutant embryos, with the *wnt3a* expression domain expanded laterally in concert (compare Figure 2.4 D'-F' with Figure 2.4 A'-C'). We conclude that the absence of functional Cdx4 is not sufficient to reduce *wnt3a* expression levels by any noticeable degree, but the *wnt3a* expression domain nevertheless shows changes.

Together, our results suggest that zebrafish Cdx4 regulates the Wnt and Fgf signaling pathways, which drive the outgrowth of the posterior body. This finding is in agreement with previous results from Cdx loss of function experiments in zebrafish [322, 321]. However, the changes in *wnt3a* expression that we find in response to Cdx4 deficiency are modest, and this is also true for the reported changes in expression of *fgf3* and *fgf8* [321]. Furthermore, interpretation of these results is challenged by the alteration of tailbud morphology in Cdx-deficient embryos. Nevertheless, given that similar relationships are observed between the Wnt and Fgf signaling pathways and Cdx2 regulation in mouse embryos [7, 303, 365, 384], I posit that Cdx factors likely form a conserved auto-regulatory loop necessary for outgrowth

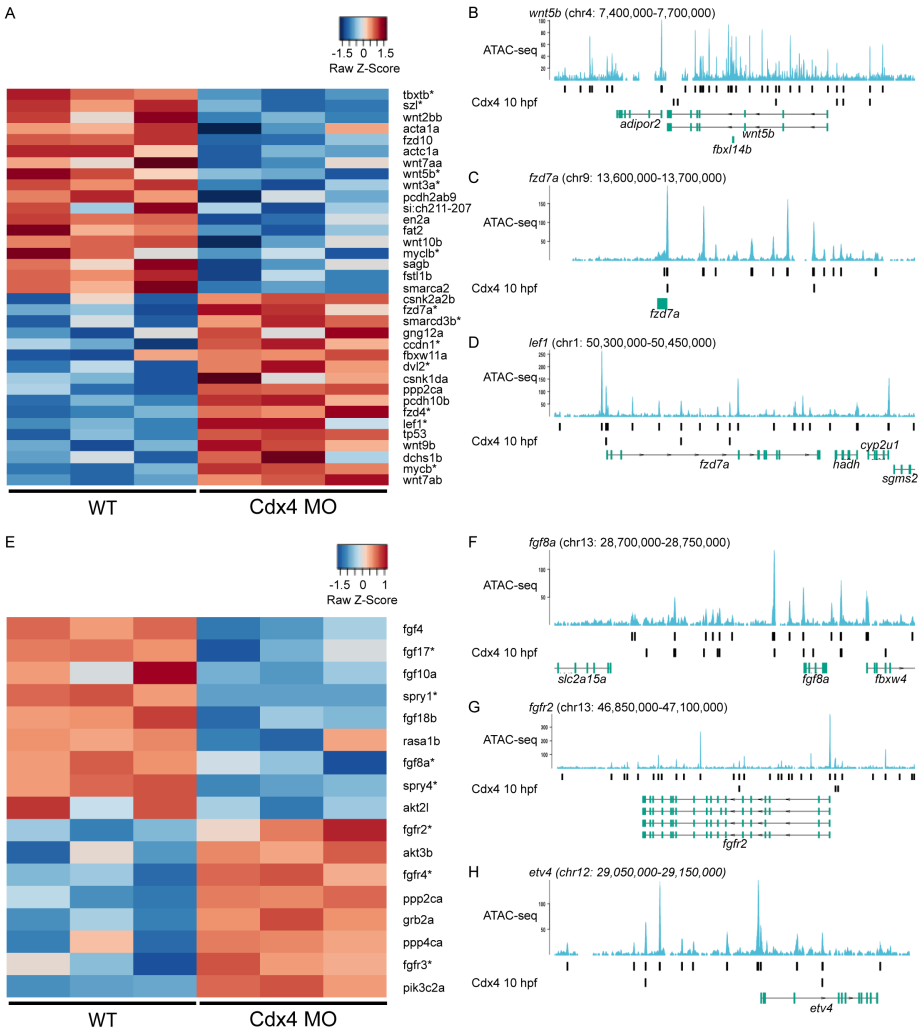

Figure 2.3: **Cdx4 regulates the Wnt and Fgf signaling pathways.** A. Heatmap of differentially expressed genes ($p < 0.05$) between WT and Cdx4 morphant embryos at 11 hpf assigned to the Wnt pathway by PantherGO. Asterisks indicate genes bound by Cdx4. B-D. Analysis of called Cdx4-Myc ChIP-seq peaks at 10 hpf (middle row) near the ligand *wnt5b* (B), the receptor *fzd7a* (C), and the transcriptional effector *lef1* (D). In all panels, the top row displays ATAC-seq profiles from *foxd3*-expressing cells with called peaks below, and the bottom row indicates Ref-Seq annotated genes. E. Heatmap of differentially expressed genes ($p < 0.05$) between WT and Cdx4 morphant embryos at 11 hpf assigned to the Fgf pathway by PantherGO. Asterisks indicate genes bound by Cdx4. F-H. Analysis of called Cdx4-Myc ChIP-seq peaks at 10 hpf (middle row) near the ligand *fgf8a* (F), the receptor *fgfr2* (G), and the transcriptional effector *etv4* (H). In all panels, the top row displays ATAC-seq profiles from *foxd3*-expressing cells with called peaks below, and the bottom row indicates Ref-Seq annotated genes.

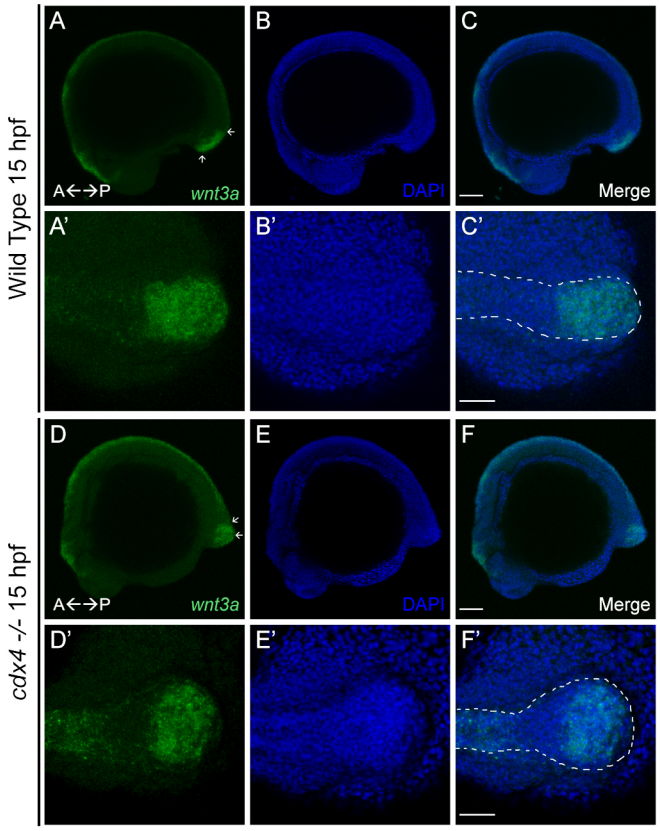

Figure 2.4: **Cdx4 regulates Wnt signaling in the zebrafish tailbud.** A-C. Lateral view of 15 hpf WT or *cdx4*$^{ch107+/-}$ heterozygous embryo shows *wnt3a* RNA transcripts detected by HCR in the dorsal spinal cord and tailbud (A), counterstained with DAPI (B), merged image of A and B (C). Small white arrows in A indicate the extent of *wnt3a* expression along the anteroposterior axis. Scale bar = 100 µm. A'-C'. Magnified dorsal view of 15 hpf WT or *cdx4*$^{ch107+/-}$ heterozygous embryo shows *wnt3a* RNA transcripts detected by HCR in the tailbud (A'), counterstained with DAPI (B'), merged image of A' and B' (C'; dashed lines outline the neural tube). Scale bar = 50 µm. D-F. Lateral view of 15 hpf *cdx4*$^{ch107-/-}$ mutant embryo shows *wnt3a* RNA transcripts detected by HCR in the dorsal spinal cord and tailbud (D), counterstained with DAPI (E), merged image of D and E (F). Small white arrows in D indicate the extend of *wnt3a* expression along the anteroposterior axis. Scale bar = 100 µm. D'-F'. Magnified dorsal view of 15 hpf *cdx4*$^{ch107-/-}$ mutant embryo shows *wnt3a* RNA transcripts detected by HCR in the tailbud (D'), counterstained with DAPI (E'), merged image of D' and E' (F'; dashed lines outline the neural tube). Scale bar = 50 µm.

of the post-cranial body across different vertebrate groups.

The interaction between Cdx factors and Wnt and Fgf signaling is also notable given the role of these pathways in patterning the NC along the anteroposterior axis. The NC is regionalized into distinct, axial specific populations [288]. Differentiation of human pluripotential stem cells (hPSCs) has shown that WNT and FGF signaling are critical for the establishment of trunk NC cell axial identity [107, 114]. Specifically, high WNT levels are crucial for determining trunk NC fate, rather than the default cranial fate [114]. Additionally, the magnitude of WNT and FGF stimuli dictate the degree of posterior identity within the trunk NC [107, 114]. Thus, the regulatory loop between Cdx and these signaling pathways may serve not only to drive posterior body outgrowth, but also to pattern the nascent NC.

2.3.4 Cdx4 and Tbxta co-bind genes of the tailbud GRN

In amniotes, much of the post-cranial body originates from a population of bipotent stem cells termed neuromesodermal progenitors (NMPs) [333, 376]. Detailed fate-mapping of the developing primitive streak and tailbud in mouse and chick uncovered distinct regions that contribute to both neural and mesodermal tissues [30, 36, 37, 315, 359, 379]. The stem cell properties of NMPs were demonstrated using both lineage tracing [202, 247] and cell transplantation [36, 37, 209] approaches. NMPs give rise to the post-cranial neural and mesodermal tissues that emerge in an anterior to posterior sequence over an extended period of embryonic development.

While NMPs have been well characterized in amniotes, examinations of this model in non-amniotes, including zebrafish, are limited and their findings remain controversial. Lineage analysis using *in vivo* imaging has revealed that bipotent zebrafish NMPs do exist at early gastrulation stages. However, these rapidly segregate into neural and mesodermal cell fates [11] before the completion of gastrulation. Additionally, amniote NMPs are defined molecularly by co-expression of the mesodermal marker *T/ Brachyury* and the neural marker

Sox2 [37, 104, 379]. Martin and Kimelman (2012) [198] described a population of zebrafish tailbud cells that similarly co-express *sox2* and the ortholog of *T/Brachyury*, *tbxta*, and, further, showed that these cells can give rise to mesoderm or neural cell types under experimental conditions, although they typically produce mesoderm in response to Wnt signaling [198]. However, these 'NMP-like' cells of the later zebrafish tailbud show significant differences from amniote NMPs. Specifically, they are mono-potent, exhibit low proliferation rates [11, 148], and contribute exclusively to the posterior-most region of the body [11]. Based on this evidence, I contend that the *sox2/tbxta*-expressing cells of the zebrafish tailbud do not exhibit the functional hallmarks of NMPs.

The points detailed above notwithstanding, it has been argued that the mechanisms governing posterior body growth are conserved across vertebrates [155, 243, 376], particularly if NMPs are viewed as a transition state from which neural or mesodermal stable states emerge [333]. Recently, studies have begun to describe the GRN that governs the formation, differentiation, and maintenance of NMPs, and Cdx factors represent key components of this NMP GRN [109]. In addition, it has been demonstrated that mouse Cdx2 and T co-regulate a core set of genes in NMPs that are necessary for axial embryonic growth [7]. We therefore investigated whether similar cooperation between Cdx and T family transcription factors might occur in the zebrafish tailbud.

During zebrafish post-gastrulation stages, *cdx4* and *tbxta* are expressed in the tailbud as well as in their separate expression domains of the spinal cord [65, 146] and notochord [310, 311], respectively. To assay whether these two transcription factor encoding genes are co-expressed in individual cells of the developing tailbud we visualized their expression patterns in 15 hpf zebrafish embryos using HCR. As expected, *cdx4* was detected in the tailbud and spinal cord, as well as at lower levels in the posterior mesoderm (Figure 2.5 A). Similarly, *tbxta* was detected in the posterior-most region of the tailbud, as well as in the notochord (Figure 2.5 B). Importantly, we found that *cdx4* and *tbxta* expression overlap in

the tailbud (Figure 2.5 C) (n= 8), and analysis of individual confocal sections confirmed co-expression in individual cells (Figure 2.5 D). This co-expression suggests that Cdx4 and Tbxta may cooperate in the regulation of target genes within the tailbud.

To investigate further whether Cdx4 and Tbxta co-regulate target genes, we analyzed available ChIP-seq datasets [244, 262] to determine whether they share commonly bound targets. Using GREAT, we identified 2,972 genes bound by Tbxta at 8-8.5 hpf, of which 1,426 were bound by Cdx4 at 10 hpf. Thus, a substantial portion of the genes bound by Tbxta are likely to also be bound by Cdx4, suggesting that these two factors may cooperatively regulate a subset of target genes. Together, these data are consistent with Cdx4 and Tbxta playing a co-regulatory role in the zebrafish, similar to the role of Cdx2 and T/Bra in the mouse.

As Cdx2 and T are integral components of the transcriptional network that underlies mouse NMP induction and differentiation [109], we investigated whether zebrafish Cdx4 and Tbxta bind zebrafish homologs of the NMP GRN. In particular, we focused on three distinct classes of genes, comprising: 1. those involved in the maintenance of the NMP state, 2. those that mediate mesodermal differentiation, and 3. those that promote neurogenic fates (Figure 2.5 E). Notably, *tbxta* and *sox2*, the orthologs of which define NMP identity at the molecular level, are both bound by Cdx4 and Tbxta at putative cis-regulatory elements, defined as regions of open chromatin by ATAC-seq of *foxd3*-expressing cells (Figure 2.5 F). The neural markers *irx3a* and *nkx1.2la* (Figure 2.5 G), and the mesodermal markers *tbx6* and *tbx16* (Figure 2.5 H), are similarly bound by Cdx4 and Tbxta. Together, these results demonstrate that zebrafish Cdx4 and Tbxta do indeed bind many of the genes involved in the induction, maintenance, and differentiation of amniote NMPs, often at the same putative cis-regulatory elements.

In summary, our findings suggest that many of the genes that comprise the genetic circuit underlying development of the post-cranial amniote body are shared targets of Cdx4 and

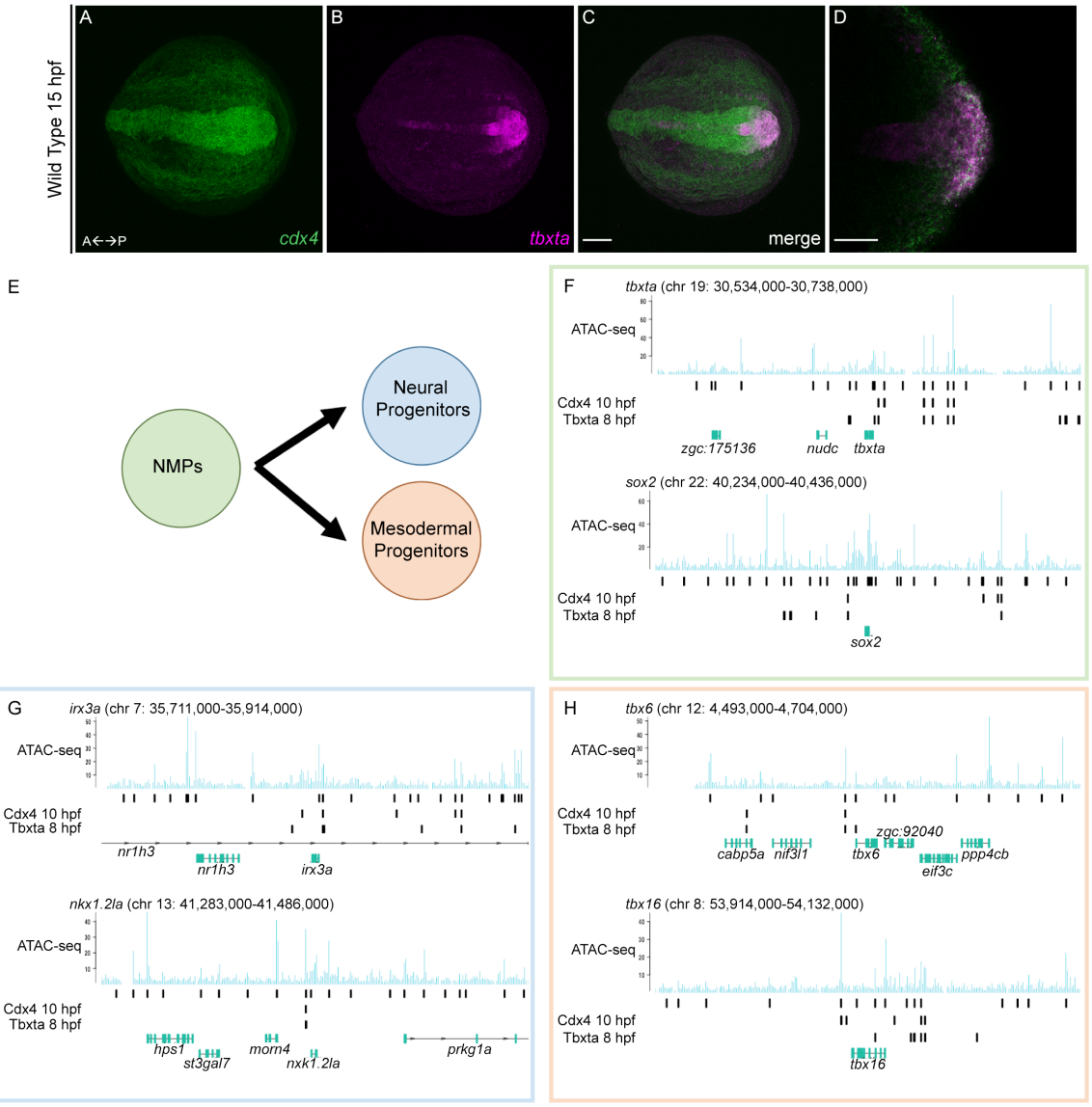

Figure 2.5: **Cdx4 and Tbxta bind near genes of the tailbud GRN.** A. Dorsal image of *tbxta* RNA transcripts in the tailbud and notochord of 15 hpf embryos detected by HCR. B. Dorsal image of *cdx4* RNA in the tailbud and spinal cord of 15 hpf embryos detected by HCR. C. Merged image reveals co-expression of *cdx4* and *tbxta* at the tailbud; scale bar = 100 µm . D. Magnified 10 µm optical section shows co-expression of *cdx4* and *tbxta* in cells of the tailbud. E. Schematic representing an "NMP-like" state. F-H. Analysis of Cdx4 and Tbxta binding near genes of the NMP-like molecular signature (F), as well as genes that drive neural (G) and mesodermal (H) differentiation. In all panels, the middle rows display called peaks of Cdx4-Myc ChIP-seq at 10 hpf and Tbxta ChIP-seq at 8-8.5 hpf. The top row shows mapped ATAC-seq in *foxd3*-expressing cells and called peaks, and the bottom row shows annotated Ref-Seq genes.

Tbxta in zebrafish embryos. We note, however, that these findings likely constitute evidence for a conserved "tailbud genetic circuit" rather than for the presence of zebrafish NMPs. To demonstrate the presence or absence of a conserved NMP-like genetic circuit in the zebrafish more conclusively, it will be necessary to investigate the GRN that governs axial outgrowth in the zebrafish tailbud at single-cell resolution. Furthermore, because NMPs are defined functionally as bipotent stem cells, it will be important to establish why the gene regulatory interactions that define NMP identity appear to be conserved in zebrafish, yet the cells of the tailbud are largely quiescent and lineage-restricted.

2.3.5 cdx4 regulates posterior body outgrowth

The truncation phenotypes observed in vertebrate embryos with abrogated Cdx function—both those previously described [46, 65, 66, 362, 363, 365, 384] and those I report here in $cdx4^{ch107-/-}$ mutants—can be explained by two non-exclusive models (schematized in Figure 2.6 A). According to the first model, Cdx function regulates differentiation and lineage allocation of tailbud cells; according to the second model, it regulates cell movements.

The model that Cdx factors regulate the differentiation of cells in the tailbud is largely supported by data from mice, where Cdx factors balance NMP differentiation. Cdx2 is necessary for sustaining the expression of T either by direct regulation through Cdx-binding sites upstream of the gene (Savory et al., 2009) or indirectly via the activation of Wnt and Fgf signals present in the niche [7, 304, 365, 384]. Additionally, Cdx factors suppress Retinoic Acid (RA) signaling in the tailbud, which is necessary for inhibiting neural differentiation [109]. Yet, Cdx factors also orchestrate the chromatin remodeling events necessary for spinal cord differentiation in mice [218]. In accord with this model, our zebrafish results indicate that Cdx4 binds putative cis-regulatory regions near *sox2* and *tbxta* (2.6 F), and thus may directly regulate their expression. In addition, we have shown that $cdx4^{ch107-/-}$ mutant embryos have altered *wnt3a* expression, and thus may exhibit an altered Wnt signaling gradient

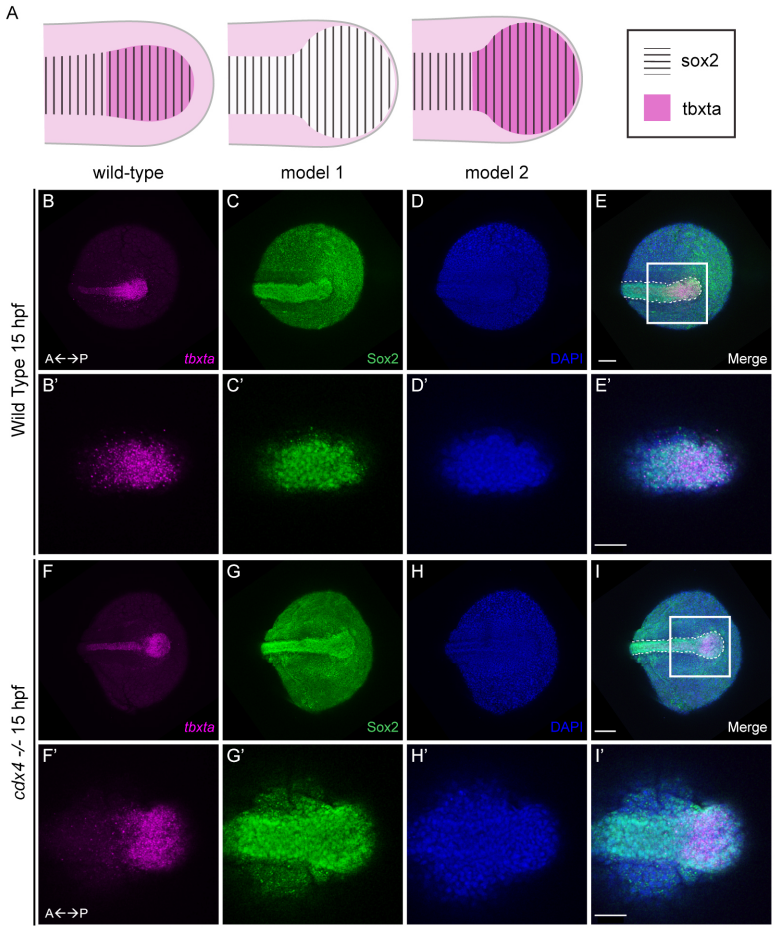

Figure 2.6: **Cdx4 does not regulate tailbud differentiation.** A. Schematic representations of the tailbuds of WT and predicted Cdx4 mutant phenotypes, as explained by models 1 and 2. B-E. Dorsal image of 15 hpf WT or *cdx4*$^{ch107+/-}$ heterozygous embryo shows *tbxta* RNA transcripts detected by HCR in the tailbud and notochord (B) and Sox2 protein detected by immunofluorescence in the tailbud and spinal cord (C), counterstained with DAPI (D). E. Merged image of B-D. Dashed lines outline the neural tube; scale bar = 100 µm. Box represents magnified region in B'-E'. B'-E'. Magnified dorsal view of 10 µm optical sections from 15 hpf WT or *cdx4*$^{ch107+/-}$ heterozygous embryo shows co-expression of *tbxta* (B') and Sox2 (C') in cells of the tailbud counterstained with DAPI (D'). E'. Merged image of B'-D'; scale bar = 50 µm. F-I. Dorsal image of 15 hpf *cdx4*$^{ch107-/-}$ mutant embryo shows *tbxta* RNA transcripts detected by HCR in the tailbud and notochord (F) and Sox2 protein detected by immunofluorescence in the tailbud and spinal cord (G), counterstained with DAPI (H). I. Merged image of F-H. Dashed lines outline the neural tube; scale bar = 100 µm. Box represents magnified region in F'-I'. F'-I'. Magnified dorsal view of 10 µm optical sections from 15 hpf WT or *cdx4*$^{ch107+/-}$ heterozygous embryo shows co-detection of *tbxta* (F') and Sox2 (G') in cells of the tailbud counterstained with DAPI (H'). I'. Merged image of F'-H'; scale bar = 50 µm.

(2.4). Previously, Martin and Kimelman (2012) [198] demonstrated that inhibition of Wnt signaling during gastrulation or segmentation stages of zebrafish development resulted in the expansion of neural fate and a concomitant loss of mesodermal derivatives. Together, the results from mouse and our own findings support a model whereby the truncation of the posterior body is due to aberrant differentiation of cells from the tailbud and improper allocation to the mesodermal or neural lineage. According to this model, the lateral expansion of the tailbud tissues could result from premature differentiation into neural or mesodermal fates.

However, an alternative model posits that the truncation phenotype is the result of impaired convergence and extension. Convergence and extension refers to a process during morphogenesis in which a tissue narrows along one dimension (here mediolaterally), as a result of cellular intercalation, and extends in another (here anteroposterior) [374]. In zebrafish, outgrowth of the posterior body is primarily driven by convergence and extension movements, rather than proliferation and differentiation of a resident stem cell population [11, 148, 243, 332]. The non-canonical Wnt pathway has been well-documented to drive convergence and extension during zebrafish epiboly [374]. Importantly, *wnt5b*, which encodes a non-canonical Wnt ligand, is bound by Cdx4 and down-regulated in Cdx4 knockdown embryos (2.3 A, B), and has been shown to be necessary for mediolateral intercalation during convergence and extension [153, 195, 371]. This second model of Cdx function is supported by our observation that the posterior-most region of the tailbud is expanded laterally in $cdx4^{ch107-/-}$ mutant embryos (2.4 D'-F'), likely due to disrupted convergence, which in turn blocks extension.

To begin to distinguish between these two models, we used whole-mount fluorescent immunolabeling and HCR to visualize expression of Sox2 and *tbxta*, respectively, in the progeny of crosses between $cdx4^{ch107+/-}$ heterozygous fish. In the tailbuds of 15 hpf WT or heterozygous sibling embryos, Sox2 immunolabeling was limited to the posterior neural

plate, located medially within the tailbud, and *tbxta* transcripts were present throughout the tailbud (2.6 B-E). Further, analysis of a 10 µm optical section confirmed that Sox2 protein and *tbxta* transcripts were co-localized in individual cells of the tailbud (2.6 B'-E'). By contrast, the overall morphology of the tailbud of $cdx4^{ch107-/-}$ mutant embryos was disrupted, and in these mutant specimens Sox2 expression was expanded laterally to encompass most of the posterior tailbud (2.6 G, I). Notably, *tbxta* was also detected in the Sox2-expressing cells (2.6 F-I, F'-I') (n= 15). Therefore, $cdx4^{ch107-/-}$ mutants exhibit a lateral expansion of the Sox2/*tbxta* expression domain, rather than the expansion of one marker and a concomitant loss of the other.

These assays for markers of neural and mesodermal fate in $cdx4^{ch107-/-}$ mutant embryos support the second model, allowing us to posit that Cdx4 regulates posterior body outgrowth via regulation of convergence and extension movements. We can further speculate that Cdx4 might regulate this process via *wnt5b* and the non-canonical Wnt signaling pathway.

The assertion that our data favor the second model lies in contrast to reports based on mouse embryo data (described above), which suggest that the truncation phenotype in Cdx-deficient embryos is due to depletion of the NMP niche, and possibly altered differentiation. This distinction likely reflects differences between these two species in the strategies by which post-cranial tissues are generated. Specifically, formation of the posterior body in mice is primarily driven by proliferation and differentiation of NMPs (reviewed in [333, 376], whereas in zebrafish cells of the tailbud are largely quiescent and growth is driven by convergence and extension of lineage-restricted cells [11, 332, 148]. As mentioned above, the two models I postulate for Cdx function are not mutually exclusive; thus, both may function, but to differing degrees in different species.

2.4 Conclusions

The induction and specification of NC cell fate in the trunk and tail regions must be tightly coordinated with the outgrowth of the post-cranial body. Here, we investigated the role of Cdx4 in integrating NC cell specification with growth of the posterior body in zebrafish embryos. Our findings suggest that Cdx4 binds near the *foxd3* locus at putative NC enhancers and regulates its expression in the posterior body. Additionally, Cdx4 and Foxd3 both bind many of the genes of the NPB module, possibly co-regulating their posterior expression. Furthermore, we have shown that Cdx4 may regulate the signaling pathways, genetic circuits, and morphogenesis that drive outgrowth of the posterior body. We conclude that Cdx4 functions as a regulatory link between these two key processes—NC cell specification and posterior body outgrowth—in zebrafish development.

These findings allow us to speculate that the molecular mechanisms that orchestrate the formation of the posterior body are largely conserved across vertebrates, despite species-specific differences. In amniotes, including mouse, chick, and humans (hPSCs), Cdx factors are critical for the establishment, maintenance, and differentiation of the NMPs that make up the posterior body. Further, NMPs may be an important source of posterior NC cells [3, 71, 93, 142, 290, 359, 379] and Cdx factors are likely critical for the specification of NC fate within this population [93, 297, 296, 295]. In teleosts, cells that meet the classic definition of NMPs have not been described and instead posterior body outgrowth is mediated by convergence and extension movements. Yet, here we have shown that zebrafish Cdx4 orchestrates the early steps in NC cell formation and regulates the signaling pathways, genetic circuit, and morphogenetic process that drive outgrowth of the posterior body. Thus, Cdx factors may have a conserved function as a regulatory nexus between NC formation and posterior body formation.

CHAPTER 3

CDX4 IS NECESSARY FOR ESTABLISHING TRUNK NC IDENTITY AND MIGRATORY BEHAVIORS

3.1 Abstract

The neural crest (NC) is a transient multipotent cell population that migrates extensively to produce a remarkable array of vertebrate cell types. NC is patterned into distinct subpopulations along the anteroposterior body axis, with cranial and trunk NC cells exhibiting axial-specific cellular behaviors during migration. However, the molecular mechanisms underlying axial-specific identity remain elusive. As the zebrafish transcription factor Cdx4 patterns the neuroepithelium along its AP axis, I investigated whether it might also play a role in regionalizing the NC. In this Chapter, I demonstrate that *cdx4* is expressed in pre-migratory NC, consistent with a functional role in this cell population. In *cdx4* mutants, segmental patterns of trunk NC cell migration are disrupted and cells fail to reach their destinations. In turn, this leads to mis-patterning of dorsal root ganglia. Further, my live-imaging analysis suggests the disrupted NC migration reflects loss of normal leader/follower cell dynamics. Finally, by using cell transplantation to generate chimeric specimens, I show that Cdx4 does not function in the paraxial mesoderm —the environment adjacent to which crest migrates— to influence NC cell behaviors. Therefore, I conclude that *cdx4* plays a critical, and likely tissue autonomous, role in the establishment of trunk NC fate and its characteristic migratory behaviors.

Attributions

The work summarized in this chapter was performed together with other members of the Prince lab. Ruby Schnirman performed a preliminary analysis of DRG development. The cell transplantation experiments summarized in Figure 3.5 were performed in collaboration

with Elaine Kushkowski. Anastasia Beiriger illustrated the schematics included in Figures 3.2 and 3.5.

3.2 Introduction

The neural crest (NC) is a transient multipotent cell population that migrates extensively and gives rise to a remarkable array of cell types including chondrocytes, melanocytes, and neurons and glia of the peripheral nervous system [166, 205, 324]. Together with cranial placodes, NC cells make up the elements of the vertebrate "new head" and likely played an important role in the radiation of vertebrates [101]. Pioneering quail-chick chimera experiments revealed that the NC comprises distinct subpopulations along the anteroposterior (AP) axis [166]. These populations exhibit important differences in cellular behaviors, differentiation potential, and their underlying transcriptional networks [288]. However, our understanding of the molecular mechanisms that pattern the nascent NC along the AP axis remains incomplete.

In addition to the striking differences in differentiation potential initially described by Le Douarin's group [166, 172, 239], cranial and trunk NC cells also exhibit distinct cellular behaviors during migration. Live imaging of NC cell migration in zebrafish embryos has revealed that cranial NC cells migrate in broad streams and dynamically rearrange without regard to their initial position. By contrast, trunk NC cells traveling along the medial pathway—between the neural tube and adjacent somites—migrate in single-cell chains and depend on a leader cell for directionality [287]. These differences may exist as a consequence of the unique challenges posed by the migratory environments encountered by cranial versus trunk NC cells. In the cranial region, zebrafish NC cells migrate ventrolaterally from the dorsal neurepithelium out into the adjacent pharyngeal arches [306], largely passing through loosely packed mesenchymal cells on route. In the trunk, zebrafish NC cells migrate along two distinct pathways: the medial pathway between the neural tube and the adjacent somites

and—commencing at a slightly later stage—a lateral pathway between the somites and the overlying ectoderm [283](reviewed in [289]). The NC cells on the medial pathway must squeeze through a narrow gap between two epithelial tissues: the neuroepithelium of the developing neural tube and the adjacent epithelialized somites. While we know navigation of trunk NC through this environment requires a specialized "leader" cell, the molecular mechanisms by which leader and follower identities are established are yet to be uncovered. Nevertheless, evidence suggests that leader/follower dynamics represent a defining property of zebrafish trunk NC cells [287].

The molecular underpinnings of NC development have been described in terms of a gene regulatory network (GRN) [196, 300, 219, 324]. Further, it has been proposed that the differences between cranial and trunk NC cells may be orchestrated by axial-specific variations in these genetic circuits. This assertion is supported by a transcriptional analysis of chick cranial and trunk NC cells, which uncovered a cranial-specific GRN that mediates axial identity and promotes chondrogenic potential [325]. Similar results were obtained in mice, where single-cell transcriptomics demonstrated important differences between cranial and trunk NC cells, in part mediated by the transcription factor Twist, which biases cranial cells towards chondrogenic differentiation [330]. Comparative analyses, using the chick cranial GRN as a starting point, suggested that the cranial NC may have evolved by progressive assembly of an axial-specific GRN [197]. However, these studies have focused largely on the cranial NC, while the question of how trunk NC identity is established remains essentially unaddressed.

The family of caudal-related homeodomain transcription factors, Cdx, are good candidates to orchestrate the establishment of trunk NC cell identity. Cdx factors are expressed in the posterior of the developing body and play a conserved role patterning the embryo along the AP axis across vertebrate model systems [383]. In zebrafish, *cdx1* and *cdx4* pattern the developing neural plate and impart its posterior identity [327]. Further, recent findings in

amniotes support a role for Cdx factors in NC cell development. In mouse, Cdx proteins directly regulate the expression of the key regulators of NC formation *Pax3*, *Msx1*, and *Foxd3* [297, 296, 295], and loss of Cdx function results in defects in the development of NC-derived melanocytes and peripheral neurons [295]. Additionally, studies of differentiation of trunk NC cells from human pluripotent stem cells (hPSCs) have suggested that Cdx2 may play a critical role in this process [93, 107, 114]. Here I have investigated a potential role for Cdx factors in establishing zebrafish trunk NC cell identity.

In this study, I have investigated the role of zebrafish *cdx4* in patterning the developing NC along the AP axis. I show that *cdx4* is expressed in pre-migratory trunk NC cells, and is required for the establishment of trunk-specific migratory behaviors. In *cdx4* mutants, the segmental patterns of trunk NC cell migration are disrupted and cells fail to reach their ventral destinations. Using time-lapse microscopy to interrogate the details of trunk NC cell behavior, I reveal that disrupted trunk NC patterning in *cdx4* mutants is likely a consequence of the loss of leader/follower dynamics. Moreover, I demonstrate that the defects in cell migration lead to mis-patterning of the dorsal root ganglia (DRG), which fail to form reiterated segmental structures. Finally, I collaborated with Elaine Kushkowski to generate genetic chimeras via cell transplantation. These experiments demonstrated that the defects in NC cell migration following loss of Cdx4 function are not due to its absence from the mesodermal cells that contribute to the somites past which the NC cells migrate. I conclude that *cdx4* plays a critical, and likely tissue-intrinsic, role in the establishment of trunk NC fate and its characteristic cellular behaviors during migration.

3.3 Results and Discussion

3.3.1 cdx4 is expressed in pre-migratory NC cells

Previous studies have reported that *cdx4* is expressed in the tailbud and spinal cord of zebrafish embryos during segmentation stages [65, 146, 322]. The anterior limit of *cdx4* expression corresponds to the boundary between the spinal cord and hindbrain, at the level of somite 3 [45]. While expression of *cdx4* in NC cells had not been described in zebrafish, *Cdx1* is detected in premigratory and early migratory NC cells in mice [220], and *Cdx2* is highly expressed throughout the differentiation of trunk NC cells from hPSCs [93, 107, 114]. Finally, because NC cells derive from the neurepithelium, where *cdx4* is already known to be expressed, I predicted that *cdx4* might be present in premigratory trunk NC cells.

To test this hypothesis, I simultaneously visualized the expression of *cdx4* and a marker of premigratory NC cells, *foxd3*, using *in situ* hybridization chain reaction (HCR) [48]. As expected, *cdx4* RNA transcripts were detected in the neural tube and tailbud of 14 hpf embryos, with an anterior limit of expression adjacent to the third somite, as well in the tailbud and lateral-most regions of the developing somites (Figure 3.1 A), consistent with previous reports. In addition, *foxd3* RNA transcripts were detected in pre-migratory NC cells located in the dorsal neural tube, as well as in the posterior somites and tailbud (Figure 3.1 B), as previously described [256]). Notably, I found that *cdx4* and *foxd3* expression overlap in the dorsal spinal cord region (Figure 3.1 C). To evaluate whether *cdx4* and *foxd3* are co-expressed in individual cells, I visualized the transcripts in transverse sections. My analysis of 5 µm optical sections confirmed that individual cells of the dorsal spinal cord do indeed express both *cdx4* and *foxd3*, (Figures 3.1 A', B', and C'), Thus *cdx4* is expressed in pre-migratory trunk NC cells, at the correct time and place to play an important role in establishing trunk NC cell identity.

The expression of *cdx4* in the developing posterior neural ectoderm [65, 146] and its role

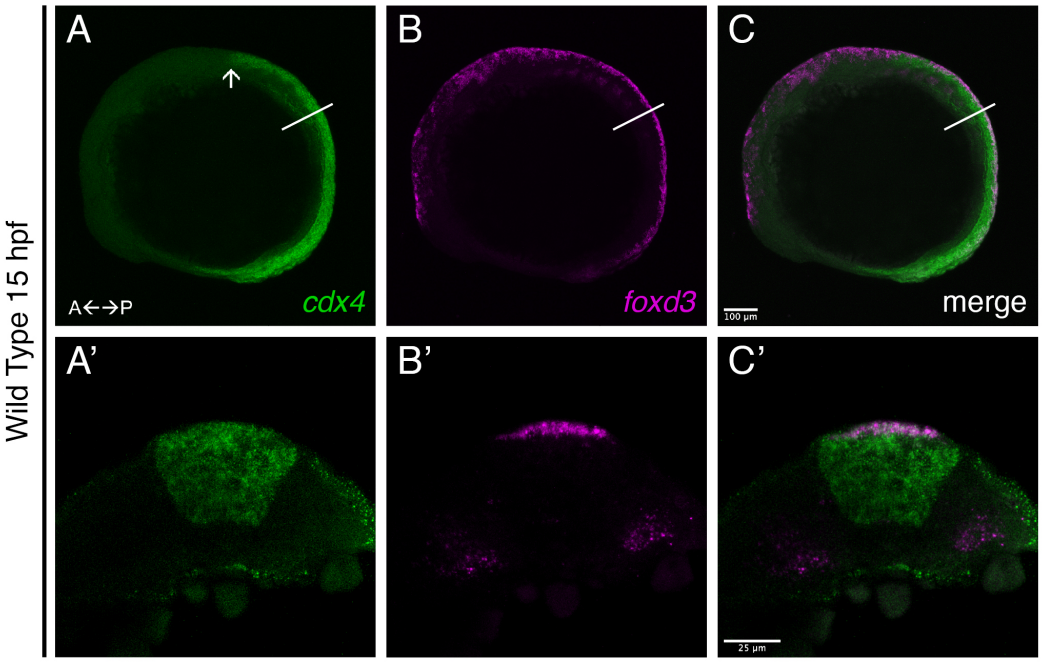

Figure 3.1: **Zebrafish *cdx4* is expressed in NC cells.** A. Lateral image of *cdx4* RNA detected in the tailbud and spinal cord of 14 hpf embryos by HCR. The anterior limit of expression corresponds to the hindbrain/spinal cord boundary (arrow). A' 5 µm optical section of *cdx4* expression in a hand-cut transverse section from the trunk (line in A). B. Lateral image of *foxd3* RNA detected in NC cells along the AP axis, the anterior somites, and the tailbud. B' 5 µm optical section of *foxd3* expression in the hand-cut transverse section from the trunk (line in B). C. Lateral image reveals co-detection of *cdx4* and *foxd3* at the dorsal neural tube. B'. 5 µm optical section of *cdx4* and *foxd3* co-expression in premigratory trunk NC cells expression in the hand-cut transverse section from the trunk (line in C).

in promoting spinal cord identity [327] have been previously described. However, whether *cdx4* is expressed in the nascent trunk NC cells, which derive from the dorsal-most part of the developing spinal cord, had not previously been assessed. Here, I show that *cdx4* RNA transcripts are present in *foxd3*-expressing premigratory NC cells of the dorsal spinal cord. Notably, this finding is further supported by RNA sequencing data on *foxd3*-expressing cells at various stages of development [190], which concur that *cdx4* is co-expressed in these cells at the stages during which NC specification is expected to occur. I did not, however, detect *cdx4* RNA transcripts in migrating NC cells. This finding again corresponds with the available data from transcriptomic analysis of *sox10*-expressing migrating NC cells, which showed that *cdx4* is not highly expressed in these cells [190]. This appears to be a minor distinction from the situation in mouse embryos, where transcripts of the related *Cdx1* gene are detected in both premigratory NC cells within the dorsal neural folds, and in early migratory NC cells [220]. My data do not address, however, whether Cdx4 protein expression might persist in the migrating zebrafish NC cells.

3.3.2 cdx4 is necessary for trunk NC cell segmental migration

One of the more remarkable differences between cranial and trunk NC cells is their distinct cellular behaviors during migration. In the trunk—but not in the cranial region—leader cells are necessary for the directed migration of NC cells traveling in segmentally arrayed chains along the medial pathway [287]. Importantly, leader and follower identities are established prior to the onset of migration and remain fixed throughout [287]. Cdx factors have previously been shown to promote posterior identity in the developing neural ectoderm [327] and we now know that *cdx4* is expressed in premigratory trunk NC cells. Therefore, I hypothesized that *cdx4* might be necessary for the establishment of trunk-specific cellular behaviors during NC cell migration.

To investigate the role of *cdx4* in trunk NC migration, I visualized the location of trunk

NC cells in the progeny of crosses between *cdx4*$^{ch107+/-}$ heterozygous fish (see Chapter 2). The embryos were co-immunolabeled with antibodies against Sox10, a NC marker, and myosin heavy chain (MHC), a somitic muscle cell marker. As expected, in wild type (WT) and *cdx4*$^{ch107+/-}$ heterozygous sibling control specimens, trunk NC cells migrated as segmental chains between the neural tube and somites (Figures 3.2 B-D, B'-D'). Notably, a single cell was present at the migratory front of each segmentally-organized chain of NC cells (Figures 3.2 B-B',D- D'; arrows). By contrast, in *cdx4*$^{ch107-/-}$ mutant embryos, the segmental distribution of trunk NC cells was disrupted; specifically, trunk NC cells were not confined to single cell-wide segmental chains and failed to reach ventral locations (Figures 3.2 F-H, F'-H'). Of note, the ventral-most position reached by the majority of trunk NC cells at this stage is adjacent to the myoseptum that divides the dorsal and ventral compartments of the somite, which also corresponds with the boundary between the neural tube and the notochord.

To analyze this phenotype in more depth, I plotted the location of migrating NC cells relative to the adjacent somite (schematized in Figure 3.2 G). In WT and *cdx4*$^{ch107+/-}$ heterozygous sibling controls (n= 3 embryos, 18 segments, 120 cells), the dorsal-most NC cells exhibited a broad distribution along the AP axis. As the NC cells migrate ventrally and approach the myoseptum (indicated by 0 on the DV axis), their AP locations become progressively more restricted, with NC cells positioned close to the center of each somite. Finally, cells migrating adjacent to the ventral portion of the somite tend to remain within a narrow spatial domain (Figure 3.2 H). In *cdx4*$^{ch107-/-}$ mutants (n= 3 embryos, 18 segments, 129 cells), however, NC cell locations are not restricted to the center of the somites, failing to converge towards the center as they near the myoseptum. Further, I found only two NC cells located adjacent to the ventral portion of the somite (Figure 3.2 I). These results indicate that *cdx4* is necessary for segmental migration of trunk NC cells along the medial pathway.

In the trunk, NC cells migrate along two pathways: a medial pathway between the spinal

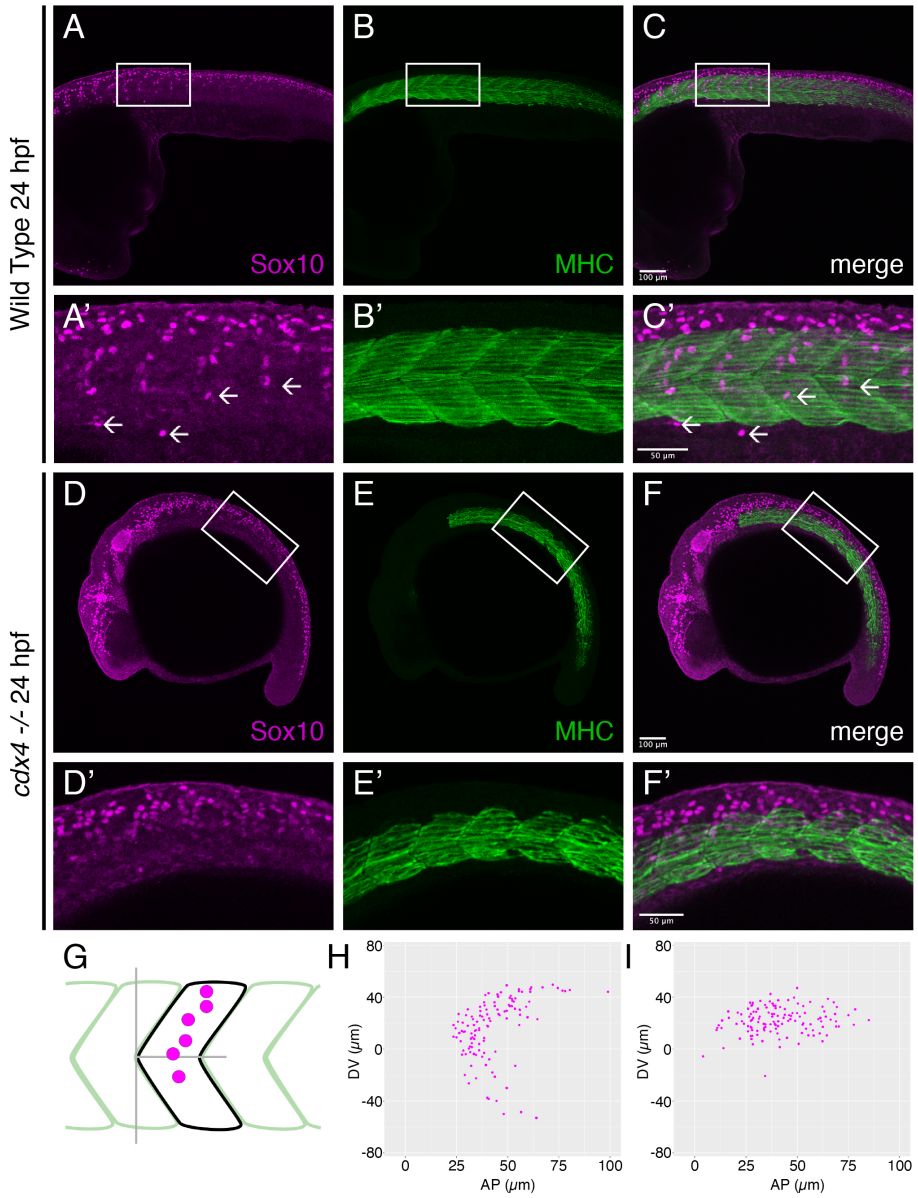

Figure 3.2: ***cdx4* mutants show impaired segmental migration of trunk NC cells.** A-C. Lateral view of 24 hpf WT embryo labeled with anti-Sox10 (NC cells, magenta) and anti-Myosin heavy chain (somites, green) antibodies reveal segmental migration pathways of trunk NC cells (arrows = individual segmental chains). A'-C'. High magnification images of boxed area from A-C. D-F. Lateral view of 24 hpf $cdx4^{ch107-/-}$ embryo labeled with anti-Sox10 (NC cells, magenta) and anti-Myosin heavy chain (somites, green) antibodies reveal defects in trunk NC cell migration. D'-F'. High magnification images of boxed area from D-F. G. Schematic representing approach for mapping the position of trunk NC cells relative to the adjacent somite. H, I. Plots of migrating trunk NC cells relative to the adjacent somite (X and Y axis units are μms) in WT embryos (H) and $cdx4^{ch107-/-}$ embryos (I).

cord and the adjacent somites, and a lateral pathway between the somites and the overlying ectoderm (Raible et al., 1992; reviewed in Rocha et al., 2020). My analysis focused on the cells that travel along the medial pathway because they exhibit region-specific migratory behaviors—including the distinction between leaders and followers—in their stereotyped, segmental migration. Here, I demonstrate that *cdx4* is necessary for proper migration of NC cells along the medial pathway. Specifically, $cdx4^{ch107-/-}$ trunk NC cells do not form segmentally organized cell chains and fail to reach the ventral portion of the embryo.

3.3.3 cdx4 regulates leader/follower dynamics

Because leader and follower dynamics are necessary for directed migration of trunk NC cells, I hypothesized that the disruption of trunk NC cell migration observed in $cdx4^{ch107-/-}$ mutants may be due to defects in establishing these two cell types. This was further supported by the fact that multiple NC cells were detected in the ventral-most positions in the trunk of $cdx4^{ch107-/-}$ mutants (Figure 3.2 D), whereas controls showed a clear single leader cell at the front of each segmental chain (Figure 3.2 A), as described by Richardson et al. [287]. Therefore, I investigated whether *cdx4* is necessary for the establishment of leader and follower cell identities in trunk NC cells.

To evaluate this proposed role of *cdx4* I visualized the migration of trunk NC cells in live embryos by single plane illumination microscopy (SPIM). For this, I generated a line of $cdx4^{ch107+/-}$ heterozygous zebrafish that also carries the *Tg(sox10:mRFP)* transgene, which labels NC cells with a membrane RFP. In WT *Tg(sox10:mRFP)* embryos, trunk NC cells migrate in single-cell chains and the first cell to migrate—termed the leader—retains its position at the front throughout migration. In fact, by tracking the position of leader cells during the migration from the pre-migratory region to a ventral position adjacent to the myoseptum (n= 17 cells, 3 embryos), I found that none of the leaders were overtaken by a follower cell during this time (Figure 3.3 A-C). However, in $cdx4^{ch107-/-}$ mutants, trunk NC

cells migrated ventrally from their site of origin, yet were unable to continue their migration past a location adjacent to the myoseptum. Additionally, I found that migrating trunk NC cells failed to converge into a single-cell stream, and instead migrated in broader streams (Figure 3.3 D-F). By tracking the first cell to migrate (n= 15, 3 embryos), I found that 12 (80%) of these were overtaken by a cell that began its migration later. Out these, 10 were overtaken prior to reaching a position adjacent to the myoseptum and 2 were overtaken after stalling at this location. Further, while leader cells in WT embryos migrate alone at the front of each chain, I found that 8 (53%) of the cells that migrated first in *cdx4*$^{ch107-/-}$ mutants migrated alongside another cell for at least 30 minutes. Therefore, these results suggest that *cdx4* is necessary for the establishment of leader and follower identities.

To understand the role of *cdx4* in orchestrating trunk-specific behaviors during NC cell migration more fully, I analyzed whether loss of *cdx4* disrupted the establishment of leader identity, follower identity, or both. To evaluate this question, I measured the size and shape of migrating trunk NC cells *in vivo*. Previously, Richardson et al. [287] reported that leader cells are larger in size than follower cells and are polarized in the direction of migration. Consistent with this previous report, I found that in WT embryos, leader cells (Figure 3.3 G) had a larger area and aspect ratio (n = 7, median area = 327.90 μm^2, median aspect ratio = 3.12) than follower cells (Figure 3.3 H)(n = 7, median area = 173.00 μm^2, median aspect ratio = 1.82) (p= 0.011 and 0.0024, respectively). In *cdx4*$^{ch107-/-}$ mutants, however, the first cell to migrate (Figure 3.3 I) (n = 9, median area = 190.68 μm^2, median aspect ratio = 2.41) did not exhibit significant differences in area (p= 0.61) or aspect ratio (p= 0.93) to cells that migrated later (Figure 3.3 J) (n = 8, median area = 166.69 μm^2, median aspect ratio = 2.32). By comparing cells from *cdx4*$^{ch107-/-}$ mutants and WTs, I found that both the first cells to migrate and those that followed in the mutants were significantly different from WT leaders, but not from WT followers, in terms of both their area and aspect ratio (summarized in Figure 3.3 K, L).

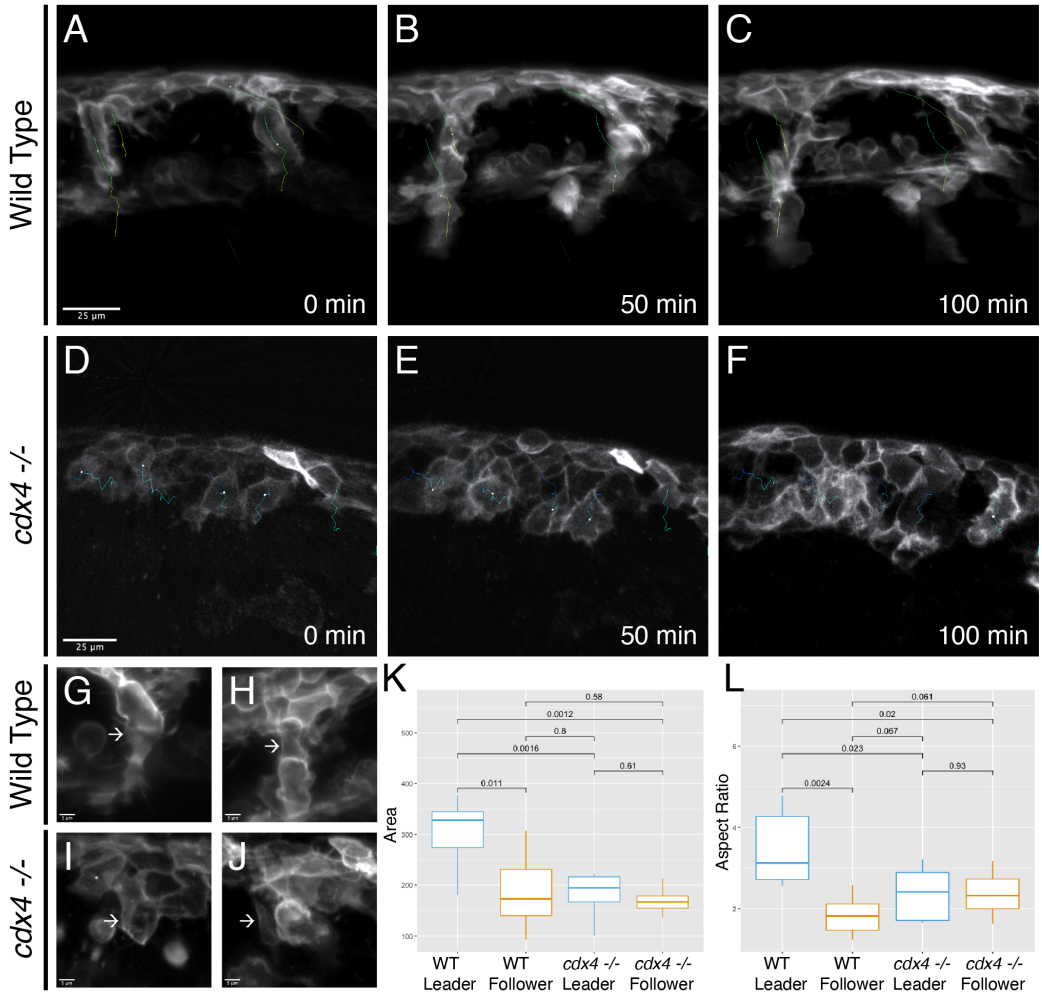

Figure 3.3: ***cdx4* mutants exhibit aberrant cell behaviors during migration.** A-J. Timelapse of trunk NC cell migration in *Tg(sox10:mRFP)* embryos visualized by SPIM reveals cellular behaviors. A-C. In WT embryos, NC cells migrate as single-cell, segmental chains. Tracking of leader and follower cells during migration shows that leader cells are not overtaken. D-F. In $cdx4^{ch107-/-}$ embryos tracking of NC cells shows that the first cells to migrate are overtaken by later-migrating cells. G, H. High magnification view in a WT specimen of the leader cell (G) and follower cell (H) . I, J. High magnification view in a $cdx4^{ch107-/-}$ mutant specimen of the first cell to migrate (I) and a later-migrating cell (J). K, L. Box plots of the area (K, units = μm^2) and aspect ratio (L) of migrating NC cells in WT and $cdx4^{ch107-/-}$ embryos.

While *cdx4* mRNA is not detected in migrating NC cells, its expression in premigratory NC cells is consistent with a potential role in regulating the establishment of leader and follower NC cell identities. In fact, Richardson and colleagues demonstrated that leader and follower identities are acquired prior to the initiation of migration [287]. Upon their experimental ablation of the leader cell, the follower cells failed to advance beyond the point of ablation. Instead, a cell from the premigratory region eventually moved to the front of the migrating chain to become a new leader [287]. Moreover, the prospective leader cells in the premigratory region could be distinguished from prospective followers based on differences in size [287]. Here, my *in vivo* imaging of trunk NC cell migration in $cdx4^{ch107-/-}$ embryos revealed that that the first NC cell to migrate—the 'leader' cell in WT embryos—is overtaken by cells that initiate their migration subsequently—the 'follower' cells in WT embryos. Furthermore, analysis of the characteristics that distinguish leader cells from followers revealed that these distinctions were absent in trunk NC cells of $cdx4^{ch107-/-}$ embryos. In fact, in $cdx4^{ch107-/-}$ specimens all of the trunk NC cells analyzed resembled follower cells, not leaders, in terms of both area and cell polarity. Consequently, I interpret my results as indicative that *cdx4* is necessary for the establishment of leader identity and the related cell behaviors that drive the highly coordinated migration of trunk NC cells.

3.3.4 Loss of Cdx4 disrupts dorsal root ganglia formation

Trunk NC cells that travel along the medial pathway give rise to the sensory neurons and glia of the DRG [166, 283, 282]. DRG are segmentally iterated structures, organized in register with the adjacent somites, and their sensory neurons transmit mechanosensory information from the periphery to the central nervous system [207, 208]. As the segmental migration of trunk NC cells along the medial pathway is impaired in $cdx4^{ch107-/-}$ mutants, I investigated whether these mutants also exhibit defects in the DRG.

To evaluate whether loss of *cdx4* disrupts the organization or formation of DRG, I vi-

sualized the distribution of DRG by immunofluorescence using an antibody against HuC/D in the progeny of crosses between *cdx4*$^{ch107+/-}$ heterozygous fish at 3 days post fertilization (dpf). In WT and *cdx4*$^{ch107+/-}$ heterozygous larvae, bilateral DRG were located adjacent to the ventrolateral portion of the neural tube one per side in each segment (100%, n= 5 embryos, 30 segments) (Figure 3.4 A-C, A'-C'), as previously described [8]. In *cdx4*$^{ch107-/-}$ larvae, however, I found defects in DRG formation, including both losses of the DRG and their mis-localization (Figure 3.4 D-F, D'-F'). Specifically, quantification of the DRG at the AP level of somites 5-10 (n= 7 embryos, 42 segments) indicated that 52% of segments lacked a DRG altogether, while 43% of segments had one cluster of HuC+ cells lateral to the spinal cord (Figure 3.4 D', arrows), and 5% had 2 or more clusters. Notably, a subset of these clusters of HuC/D expression were not detected at the stereotypical location of DRG (Figure 3.4 D', arrowhead). Together, these results suggest that loss of *cdx4* impairs the formation of DRG, likely as a consequence of the defects I observed in the segmental migration of NC cells along the medial pathway.

3.3.5 Cdx4 function is not required in the somites for proper trunk NC cell migration

In zebrafish embryos, the behaviors of trunk NC cells that migrate along the medial pathway are influenced by both NC cell-extrinsic and -intrinsic interactions. Specifically, the segmental pattern of trunk NC cell migration in regulated by the slow muscle precursors—adaxial cells—in the adjacent somites [133], possibly via signaling interactions mediated by MuSK and its ligand Wnt11 [12]. On the other hand, cell-cell contacts between NC cells are equally critical for directing cell migration. When these contacts are disrupted, either by laser ablation [287] or loss of the proto-cadherin encoding gene *pcdh10a* [373], migration along the medial pathways is halted.

Given that *cdx4* expression is not restricted to trunk NC cells, I considered the possibility

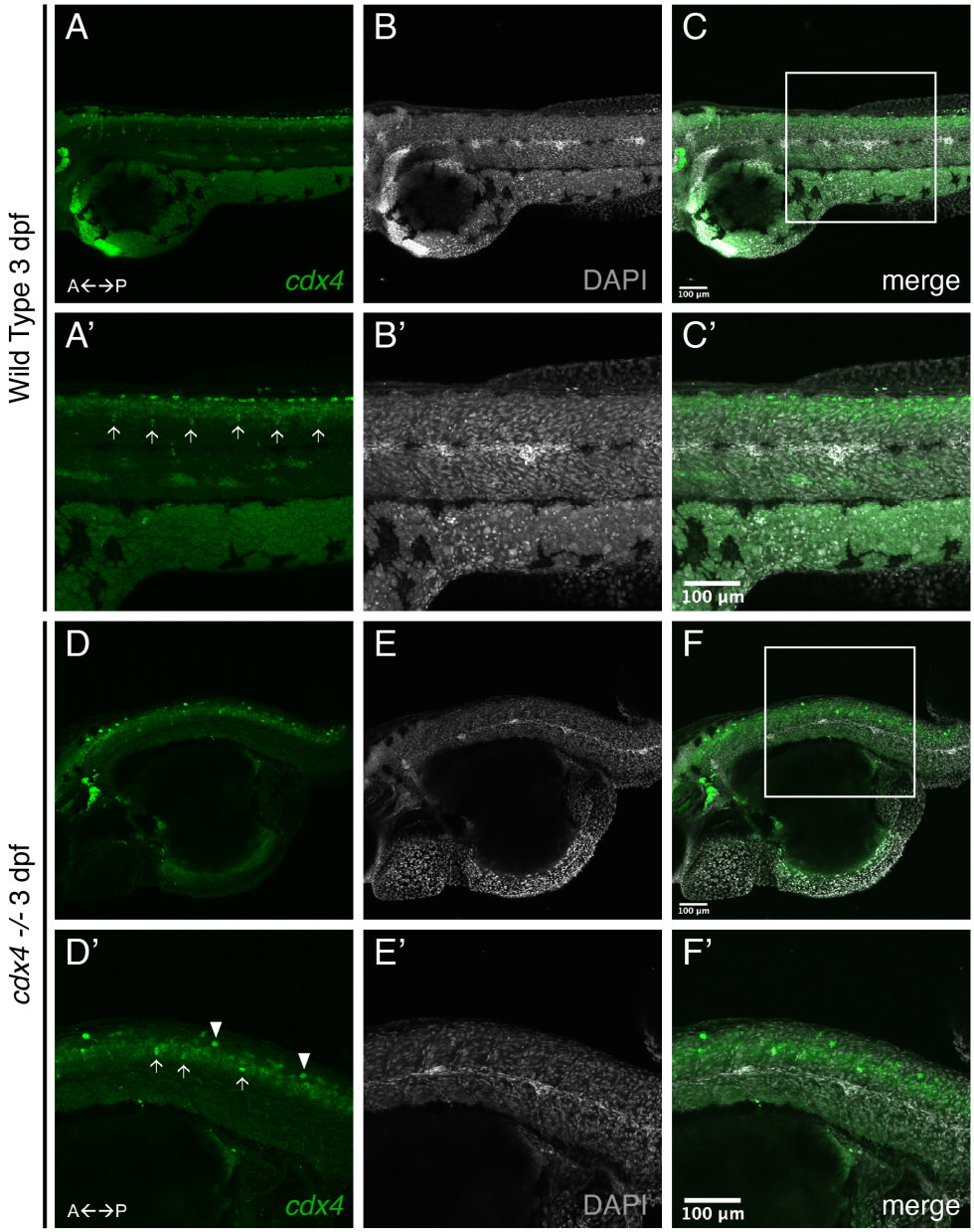

Figure 3.4: **DRG formation is disrupted in *cdx4* mutants.** A-C. Lateral view of 3 dpf WT embryo labeled with anti-HuC/D antibody (A) and DAPI (B) reveals segmental organization of sensory neurons of the DRG. C. Merged image of A and B. A'-C'. Higher magnification image of the boxed region in C (Arrows = DRG). D-E. Lateral view of 3 dpf $cdx4^{ch107-/-}$ embryo labeled with anti-HuC/D antibody (D) and DAPI (E) reveals defects in DRG formation. F. Merged image of D and E. D'-F'. Higher magnification image of the boxed region in F shows that while DRG are present in some segments (arrows), they are often lacking. Arrowheads indicate HuC-positive clusters that are not located at the stereotypic position for normal DRGs.

that the defects I observed in *cdx4*$^{ch107-/-}$ trunk NC migration might result from Cdx4 action beyond the NC. In addition to expression in the spinal cord, *cdx4* RNA transcripts are also present in the margin during gastrulation stages [65, 146] and in the tailbud (Figure 3.1 A) during segmentation stages, both of which contribute to the developing somites. Further, loss of Cdx function has previously been shown to impact mesoderm development, including the position and development of the pronephros [377] and lateral plate mesoderm derivatives [281]. Consistent with previous reports, I find that the shape and size of the paraxial mesoderm-derived somites is altered in *cdx4*$^{ch107-/-}$ mutants ([65] and see Figure 3.2). As NC cells migrate between the somites and the neural tube [283], and somite cells are in turn known to play an important role in regulating NC migration [133, 12, 13], it is possible that loss of Cdx4 function in the mesoderm could disrupt the environment through which trunk NC cells migrate, and thus disturb their behavior in a non-cell-autonomous manner.

To test the possibility that Cdx4 function in the paraxial mesoderm-derived somites is necessary to allow normal NC cell migration, I collaborated with Elaine Kushkowski to generate chimeric specimens via a cell transplantation approach [126]. We elected to use a morpholino knock-down approach for these experiments because the transplants were performed prior to the stage at which mutants can be phenotypically recognized; the knockdown approach ensured Cdx4-deficiency in all experimental specimens. Importantly, Cdx4 MO-injected embryos fully recapitulate the phenotype of *cdx4* mutants, and have been extensively used in previous studies [45, 65, 66, 122, 262, 281, 322, 321, 327]. Donor embryos were also labeled with 10 kDa lysinated Alexa Fluor 647 dextran, to enable tracking of donor-derived cells within the chimeras. Additionally, the host embryos were double transgenics, carrying both the *Tg(sox10:mRFP)* transgene to label NC, and the *Tg(His-GFP)* transgene to label all nuclei.

We first generated chimeras in which Cdx4 function is absent from the somites but intact in the NC cells. Specifically, cells from blastula-stage (4 hpf) embryos injected with

morpholinos targeted against *cdx4* (Cdx4 MO) were transplanted into WT blastula-stage hosts (Figure 3.5 A). By transplanting Cdx4-deficient donor cells into the margin, the region of the fate map that contributes to paraxial mesoderm, we were able to reliably generate chimeric embryos in which the somites were Cdx4-deficient, while the NC cells remained WT. At 25 hpf the chimeric embryos appeared morphologically normal (Figure 3.5 C, C') and many donor-derived cells were present in the somites (Figure 3.5 D, D'). However, despite the presence of Cdx4-deficient cells in the somites, trunk NC cell migration was unaffected, as shown by the normal organization of the segmental NC streams (Figure 3.5 A, A', E, E'). In fact, unperturbed trunk NC cell chains were evident in all segments, including those where donor-derived cells made up >20% of the somitic area (n= 9 embryos, 37 segments). I conclude that loss of Cdx4 function in the mesoderm cells that generate the somites does not disrupt trunk NC cell migration.

In reciprocal experiments, we generated chimeras in which Cdx4 function was exclusively limited to the somites. To this end, labeled cells from WT blastula-stage donor embryos were transplanted into the margin of blastula-stage, Cdx4 MO-injected hosts (Figure 3.5 F). As expected, the chimeric embryos exhibited a shortened tail and aberrant morphology, consistent with the overall mutant phenotype of the host embryos (Figure 3.5 H,H'). While donor-derived cells, in which Cdx4 function was intact, are present in the somites (Figure 3.5 I,I'), trunk NC cell migration remained aberrant in all cases (n= 5 embryos, 22 segments) (Figure 3.5 G, G', J, J'). Trunk NC cells were only found adjacent to the ventral half of the somite in a single segment, (4.5%), and even in this case the NC did not form a typical WT stream. I conclude that restoring Cdx4 function in the cells that generate the somites is not sufficient to rescue the defects in trunk NC migration observed in Cdx4-deficient embryos.

In summary, based on the transplantation experiment results, I conclude that Cdx4 function is not required in somitic cells for proper segmental migration of trunk NC cells. While these data do not directly address whether *cdx4* acts cell-autonomously in NC cells,

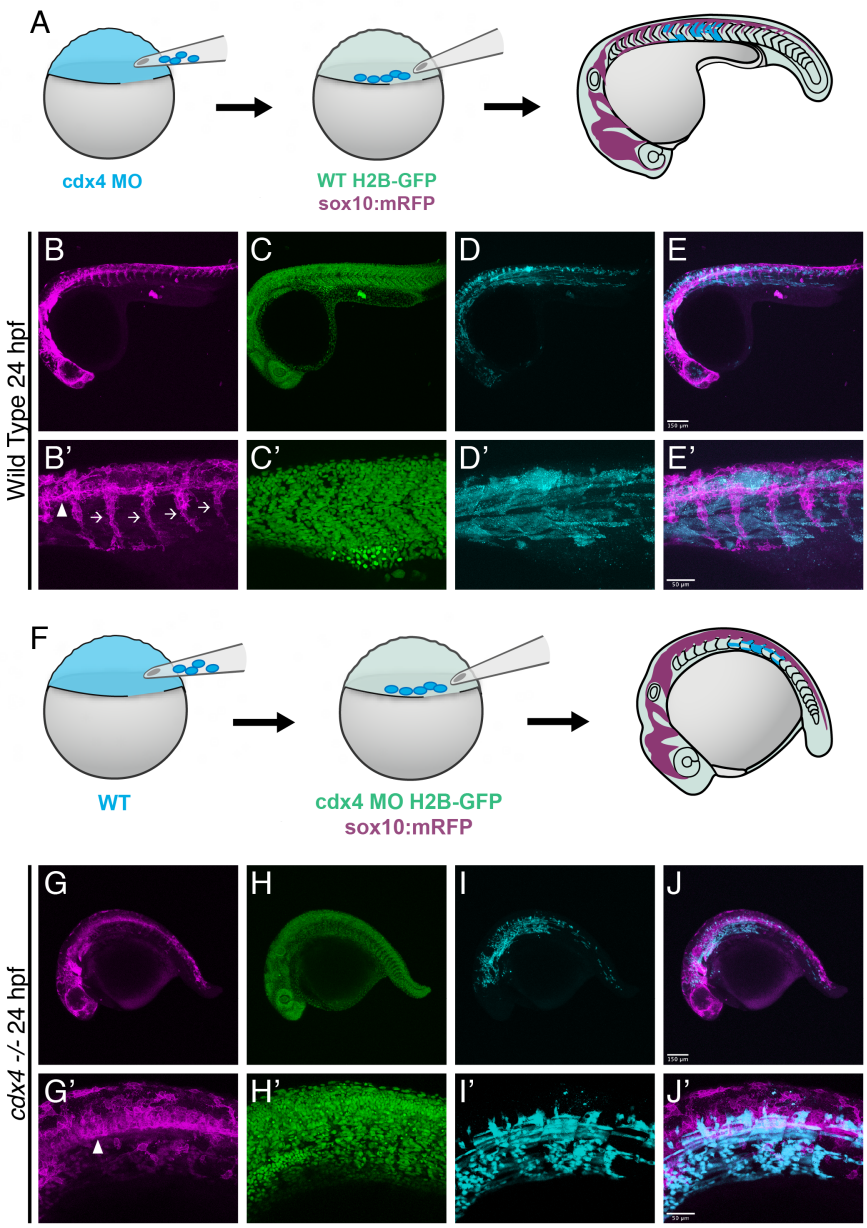

Figure 3.5: **Cdx4 function is not necessary in the somitic mesoderm.** A. Schematic for the transplantation approach to generate chimeric embryos with Cdx4-deficient cells in the somites B-E. Lateral image of NC migratory streams (magenta), cell nuclei (green) showing the overall morphology of 25 hpf chimeric embryos with Cdx4-deficient cells (cyan) in the somites. B'-E'. Higher magnification images of somites 4-8 from B-E. F. Schematic for the transplantation approach to generate chimeric Cdx4-deficient embryos with WT cells in the somites G-J. Lateral image of trunk NC cells (red), cell nuclei (green) showing the overall morphology of 25 hpf chimeric Cdx4-defieicnt embryos with WT cells (cyan) in the somites. G'-J'. Higher magnification images of somites 3-7 from G-J.

the results are nevertheless fully consistent with that hypothesis.

3.4 Conclusions

The NC is regionalized into distinct subpopulations along the anteroposterior axis that exhibit differences in differentiation potential, underlying genetic circuits, and cellular behaviors. In zebrafish, cranial and trunk NC cells rely on unique behaviors during their directed migration from the dorsal neural tube into the body. While cranial NC cells regularly interchange positions at the leading edge, trunk NC cells have well-defined and non-interchangeable leader and follower identities that are established prior to the onset of migration. Here, I show that *cdx4* is expressed in premigratory NC cells in the trunk, and in *cdx4* mutants trunk NC cells fail to form the characteristic reiterated single-cell chains that migrate along the medial pathway. Moreover, by visualizing trunk NC cell migration *in vivo*, I found that *cdx4* is necessary for establishing leader identity. I also demonstrated that *cdx4* mutants exhibit defects in the development of sensory neurons of the DRG ganglia, possibly secondarily to their impaired migration. Finally, the results from the analysis of chimeric embryos suggest that Cdx4 function is not required in the adjacent somitic cells for proper migration of trunk NC cells. Therefore, I conclude that *cdx4* is necessary for regulating the trunk-specific cellular behaviors that underlie directed migration along the medial pathway.

CHAPTER 4

MATERIALS AND METHODS

4.1 Animal Husbandry

Zebrafish (*Danio rerio*) were maintained in accord with IACUC-approved protocols. Embryos were maintained in E3 solution (in mM: 5.0 NaCl, 0.17 KCl, 0.33 CaCl, 0.33 MgSO4) and staged according to standard guidelines [156]. Embryos were obtained from crosses of adult fish stocks of mutants and/or transgenics. Transgenic zebrafish lines *Tg(-7.2sox10: mRFP)vu234* (referred to as *Tg(sox10:mRFP)* [158] and *Tg(h2az2a:h2az2a-GFP)* (referred to as *Tg(His-GFP)*) [268] have been previously described. The *cdx4* mutant $cdx4^{ch107}$ line is characterized by a missense mutation and precocious stop (nonsense) codon, as described below.

4.2 Generation of the $cdx4^{ch107}$ allele

A guide RNA for CRISPR-based mutagenesis was generated by annealing a *cdx4*-specific oligo, 5′C-GTGTGGAAACAAAGTTCTGTGG-3′C, to the trRNA sequence followed by *in vitro* transcription as described in [98]. *cas9* mRNA was *in vitro* transcribed from plasmid pT3TS-nCas9n [141] and purified as described [98]. 100 pg *cdx4* sgRNA and 300 pg *cas9* mRNA were co-injected in a 1.25 nl volume into early 1-cell stage wildtype (*AB) zebrafish embryos. Injected F0 specimens were raised to adulthood and genotyped to identify genetically mosaic cdx4 mutant fish. F0 mutation carriers were then outcrossed to generate individual *cdx4* mutant F1 fish. An F1 *cdx4* mutant carrier, containing a 1 base pair deletion directly followed by a 5 base pair insertion after nucleotide 84 in exon 1, was identified and designated as $cdx4^{ch107}$. $cdx4^{ch107}$ is predicted to produce missense mutations after amino acid 28 and a precocious stop (nonsense) codon at amino acid position 102. The $cdx4^{ch107}$

F1 founder was outcrossed to produce the F2 generation, and adult heterozygous F2 siblings were inbred to produce *cdx4*$^{ch107-/-}$ homozygous mutant embryos for study.

4.3 ChIP-seq Analysis

ChIP-seq peak files were obtained from NCBI GEO: Cdx2, GEO: GSE84899; Cdx4, GEO: GSE48254; Tbxta, GEO: GSE84619; and Foxd3 GEO: GSE106676. All zebrafish datasets were converted to the genome assembly danRer7 using the liftOver tool [125] to enable comparison. GR[210] was used to annotate peaks to the single nearest gene within 100 kb and perform GO term analysis. Further analysis of bound genes was performed using custom R scripts (available on request). ATAC-seq data was also obtained from GEO: GSE106676 and coverage was converted to danRer7 using the liftOver tool in the 'rtracklayer' package [165]. ChIP-seq and ATAC-seq data were visualized using the 'Gviz' package in R [115].

4.4 Hybridization Chain Reaction (HCR)

Antisense DNA probes were designed against the full-length zebrafish *cdx4*, *foxd3*, *tbxta*, and *wnt3a* mRNA sequences as described by Choi et al. (2018) and purchased from Molecular Instruments. Embryos were fixed with 4% PFA (PFA; Sigma) at 4°C overnight and then stained as previously described [48]. Embryos were mounted in 1% low melting agarose for imaging.

4.5 Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde (PFA; Sigma) and immunohistochemistry was performed as previously described (Prince et al., 1998) using the following primary antibodies: rabbit anti-Sox2 (1:500; GeneTex, GTX124477, lot # 42676), and the secondary antibody goat anti-rabbit Alexa Fluor 488 (1:500; Invitrogen, A11008, lot # 1073082). rabbit

anti-Sox10 antibody (1:100, GeneTex, GTX128374), mouse anti-myosin heavy chain (1:100, Developmental Studies, Hybridoma bank, IA, USA, A4.1025), and mouse anti HuC/D (1:50, Molecular Probes A-21271). The following secondary antibodies were used: goat-anti mouse highly cross-adsorbed Alexa Fluor Plus 488 (Molecular Probes A32723), goat anti-rabbit highly cross-adsorbed Alexa Fluor 488 (Molecular Probes A11034), goat anti-rabbit cross-adsorbed Alexa Fluor 546 conjugate (Molecular Probes A11010). Embryos were also stained with DAPI (R37606). Embryos were then mounted in 1% low melting agarose for imaging.

4.6 Confocal Image Acquisition

For assays in fixed specimens, embryos were fixed in 4% PFA at 4°C overnight. Following overnight fixation, embryos were washed in 1X PBS five times for 5 min each. For long-term storage of embryos, embryos were washed in 30%, 60% and 100% methanol (diluted in 1X PBS) and stored in 100% methanol at -20°C. If stored in 100% methanol, embryos were progressively washed in 60%, 30% methanol as well as 1X PBS + 0.1%Tween-20 before mounting or staining. For transverse section analysis, 50-100 µm sections were cut by hand from the trunk of embryos in PBS and embedded in 1% low-melt agarose (MidSci IB70051 St. Louis, Missouri) for subsequent imaging.

Images were collected using an upright Zeiss LSM710 confocal microscope with a Plan-Apochromat 10x/0.45 (working distance: 2.1mm) objective. Green fluorescent dyes (Alexa Fluor 488) were excited by a 488 nm laser. Red fluorescent dyes (Alexa Fluor 546) were excited by a 543 nm laser. DAPI dye was excited using a 405 nm laser. For a single fluorophore or a combination of fluorophores, spectral unmixing was used to define emission fluorescence range. Images were acquired and saved as .czi files using Zen (Zeiss) software and processed with FIJI (Schindelin et al., 2012).

4.7 Single Plane Illumination Microscopy

Zebrafish embryos were staged to 14 hpf and mounted in 1% low melting agarose (MidSci IB70051 St. Louis, Missouri) dissolved in E3 medium and 0.2 mg/ml tricaine using glass capillaries (Zeiss, 701904). Embryos were incubated at 28.5°C during data collection. Images were captured with a Zeiss Lightsheet Z.1 single-plane illumination microscope (Carl Zeiss Microscopy) with tandem PCO.edge sCMOS cameras (PCO.Imaging, Kelheim, Germany) and Zeiss Zen imaging software. A 20×/1.0 long working distance detection objective was used alongside a pair of 10×/0.2 dry illumination objectives, and the excitation sheet was narrowed to 2.0 µm. Volumes were acquired every 5 min between 15 and 21 hpf, with 119 ms exposure per slice for both green (488 nm, 7.5%) and red (561 nm, 7.0%) channels. Cell tracks were manually reconstructed using Imaris.

4.8 NC cell quantifications

To plot the position of migrating NC cells relative to the adjacent somite, the coordinates for the Sox10 label and the vertex of each somite were obtained in FIJI. The position of each Sox10 point was then normalized relative to the position of the vertex of the adjacent somite in R. Plots of the distribution and 2D density of these points were then generated using ggplot2 [372]. FIJI was also used to measure the cell area and aspect ratio. Box plots of the cell areas and aspect ratios were generated using ggplot2 and t-tests were performed using ggpubr (https://rpkgs.datanovia.com/ggpubr/index.html) in R.

4.9 Transplants

Cell transplantation experiments were performed as previously described [126]). To knock-down Cdx4 function, antisense *cdx4* morpholino (20 ng) (Davidson et al., 2003) oligonucleotides (Gene Tools LLC) were injected into one-cell stage donor or host embryos, as de-

scribed [327]. In addition, donor embryos were injected with 10 kDa lysinated Alexa Fluor 647 (Molecular Probes). Cells from blastula-stage (4 hpf) donor embryos were transplanted into the margin of blastula-stage hosts. Chimeric embryos were allowed to develop to 25 hpf and fixed in 4% PFA at 4°C overnight before further analysis.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

In the previous Chapters 2 and 3, I have presented evidence for the role of zebrafish *cdx4* in linking the development of NC cells with outgrowth in the posterior body and highlighted its function in trunk NC cell migration. In the following section, I contend that these data collectively support the model that Cdx4 is an early NC specifier in the posterior of the developing embryo. I then propose additional experiments to test this model and build on my findings to date.

5.1 *cdx4* functions as a NC specifier in the posterior body

My results indicate that *cdx4* is expressed at the appropriate time and place to act as a NC specifier. Here, I demonstrated that *cdx4* is expressed in *foxd3*-expressing premigratory NC cells in the trunk present at the dorsal neural tube. In addition, *cdx4* is also expressed in the developing tailbud, including a region that contains *foxd3*-expressing cells. While the expression of *cdx4* is not limited exclusively to prospective NC cells, these expression patterns are consistent with those observed for other early NC specifier genes. Moreover, I did not detect *cdx4* transcripts in migrating NC cells, which parallels the published descriptions of other early NC specifiers [152].

The early NC specifier genes function to promote NC cell fate in a region between the neural plate and the non-neural ectoderm termed the neural plate border (NPB). However, the NPB does not only give rise to NC cells, but also contributes to placodal cells in the cranial region, epidermis, and the dorsal neural tube [272, 346]. During gastrulation and neurulation, the NPB border emerges as a distinct territory defined by the overlapping expression of several transcription factors [272], which include the early NC specifier genes that make up the NPB module of the NC GRN [324]. Critically, the expression of most of

these genes, in a variety of species, often extends beyond the NPB region [272], as discussed in more detail below.

Studies in *Xenopus* embryos were the first to determine that the transcription factors *Msx1* and *Pax3* integrate FGF, WNT, and BMP signals to promote NC specification at the NPB [228]. However, while the expression of *Pax3* is restricted to the NPB region, the expression of *Msx1* extends all the way from the ventral side of the embryo to the edge of the neural plate [228]. In fact, *Xenopus Msx* genes play a dual role, mediating epidermal induction [335] in addition to NC specification [356]. Similar expression of *Msx1* in the ventral non-neural ectoderm as well as the NPB has been described in chick embryos [152], where *Pax3* and *Pax7* are largely restricted to the NPB during gastrulation and early neurulation [17, 152], although they are later present at the dorsal neural tube [261]. In zebrafish, *msx* genes are largely expressed in bilateral stripes at the edges of the neural plate at the beginning of neurulation [269], whereas *pax3* and *pax7* exhibit a broader expression domain, which extends into the neural plate and later the dorsal neural keel [316].

Another key specifier of NC cell fate is the transcription factor Tfap2a [68, 192]. Recent genomics analysis in chick embryos has revealed that Tfap2a heterodimerization with the paralogous proteins Tfap2c and Tfap2b drives the genetic programs that orchestrate NPB formation and NC specification, respectively [293]. Yet, *Tfap2a* expression initially extends beyond the NPB and into the non-neural ectoderm and only later becomes restricted to the neural folds [293]. By contrast, the transcription factor *Zic1* acts in combination with *Pax3* to regulate NC cell specification [129]. Yet, *Zic1* is expressed beyond the NPB region and into the neural plate [129]. Moreover, *Zic1* is also necessary for the development of cranial pre-placodal cells [129], as well as the activation of pro-neural genes that drive neural and neuronal differentiation [226].

Thus, the NPB border does not represent a single cell population defined by a restricted set of marker genes. Instead, the NPB consists of a heterogeneous region where a suite of

transcription factors, with both restricted and more expansive expression domains, overlap. Within this region, feed forward interactions between these transcription factors stabilize and refine the NPB domain [272] and drive the expression of subsequent components of the NC GRN [324]. I conclude, therefore, that although the expression of *cdx4* is not limited to the neural plate border, nor later to the dorsal neural tube where NC cells are specified, its expression domain is nevertheless consistent with a role as an early NC specifier.

NC specifier genes are defined functionally by their role in regulating the expression of other genes involved in the establishment of NC cell fate [277]. Based on the results presented in this thesis, I propose that Cdx4 may regulate the expression of early NC specifier genes, namely those of the NPB module. Here, I have shown that Cdx4 binds at four regions of open chromatin near the *foxd3* locus that have been previously demonstrated to act as NC-specific enhancers [190]. Moreover, in $cdx4^{ch107-/-}$ mutants, *foxd3* expression is not detected in the tailbud and is also downregulated at the dorsal neural tube. Together, these results suggest that *cdx4* is necessary for normal expression of *foxd3* in the posterior body.

The finding that Cdx4 regulates the expression of *foxd3* parallels results observed following loss of other early NC specifiers. In zebrafish, Prdm1a directly binds to an enhancer of *foxd3* to activate its expression [275]. In *prdm1* mutants, expression of *foxd3* is downregulated leading to a reduction in the number of NC cells [124]. Moreover, injection of *foxd3* mRNA rescues defects in NC cell development caused by morpholino knockdown of Prdm1a. Similarly, injection of morpholinos targeted against *tfap2a* and *tfap2c* into zebrafish embryos eliminates the expression of *foxd3* [182], suggesting that these genes also function upstream of *foxd3*. Finally, the regulation of *foxd3* by early NC specifiers has been well-documented in other vertebrate model systems, as summarized by Prasad et al. (2019) [277] and Simões-Costa and Bronner (2015) [324]. Thus, the regulation of *foxd3* expression in the posterior body by Cdx4 represents a hallmark of early NC specifier gene function. The NC GRN is also characterized by cross-regulation and feedforward loops, as postulated by Simoes-Costa

[324], and subsequently demonstrated *in vivo* by Tatjana Sauka-Spengler's lab [185, 375]. In the case of *foxd3*, it has also been shown to regulate the expression of early NC specifiers, including *tfap2a* and *prdm1a* [190].

Finally, the analysis of previously-reported ChIP-seq datasets presented in Chapter 2, suggests that Cdx4 and Foxd3 both bind near 13 out of 17 genes that make up the NPB module. In fact, detailed examination of these loci revealed that these two transcription factors may bind at the same putative enhancers. Based on these results, I speculate that Foxd3 and Cdx4 may function co-operatively to activate the expression of these NC specifier genes. Further, both Cdx4 and Foxd3 bind near 8 out of 15 genes of the premigratory NC module, although generally at different regions of open chromatin. While the possible regulatory interaction between Cdx4 and Foxd3 has yet to be experimentally evaluated, it reflects a common trend observed in the NC GRN. This has been demonstrated by the dissection of the regulatory logic of Foxd3 [326] and Sox10 [23] in chick embryos, which revealed that the NC enhancers that drive expression of these genes are regulated by the combination of various NC specifier genes. More recently, genomic and epigenomic approaches used to investigate the role of Foxd3 in zebrafish embryos revealed that transcription factor binding motifs for other NC specifier genes, including those of the Sox and Pax families, are highly enriched at FoxD3-bound genomic regions [190]. Thus, co-regulation between the transcription factors that make up the NC GRN, as proposed here between Cdx4 and Foxd3, appears to be a common feature of this genetic circuit.

A model in which Cdx factors function to specify NC in the posterior has been previously postulated based on results from hPSC differentiation into trunk NC cells. Specifically, in this *in vitro* system, Cdx2 is expressed at high levels during the generation of trunk NC cells [93, 114, 107]. Consistent with this model, a study showing that mouse Cdx factors function within the trunk NC GRN was published during the course of my dissertation research. In this study, Sanchez-Ferras and colleagues (2016) [295] conditionally expressed

a dominant-negative Cdx protein in the murine neural tube and premigratory NC cells. These mice exhibited defects in pigmentation and enteric nervous system development [295], characteristic of impaired NC cell development. Further, the authors also showed that Cdx factors directly regulate the expression of other NC specifier genes, namely *FoxD3*, *Msx1*, and *Pax3* [295]. These findings, in addition to a previous report that mouse Cdx promotes the expression of *Pax3* at the neural plate border downstream of Wnt signaling [297] together with the NPB transcription factor Zic2 [296] strongly suggest that Cdx factors regulate the expression of NPB genes and thus function as NC specifier gene. The results I present in this thesis are therefore in agreement with reports from other experimental models. Collectively, they indicate that Cdx factors play an essential role in mediating the early regulatory events that result in NC cell specification across distant vertebrates.

To understand the role of *cdx4* as a NC specifier more fully, it will be important to test the hypothesis that it regulates the expression of NPB genes in the posterior of the embryo. To test this model, I propose experiments to visualize the expression of NPB genes by HCR in the progeny of crosses between $cdx4^{ch107+/-}$ heterozygous fish. In particular, the NPB genes *pax3a* and *msx1a* are promising candidates, as they are not only expressed at the dorsal neural tube but also in the developing tailbud. If my hypothesis is correct, I predict that the expression of these genes in the posterior body will be markedly reduced in $cdx4^{ch107-/-}$ mutant embryos relative to WT or heterozygous sibling embryos. Additionally, it will be important to analyze the expression of these NPB genes, as well as *foxd3*, in embryos injected with morpholinos against both *cdx1a* and *cdx4* (Cdx1a/4 MOs), because Cdx factors have been demonstrated to function redundantly in the development of the posterior body in both zebrafish [45, 65, 66, 327], and mouse [46, 362, 363, 365, 384]. If the Cdx factors also function redundantly in early development of the NC, then double knockdowns will likely serve to exacerbate reductions in NPB gene expression.

In addition, future experiments are needed to characterize the regulatory interactions

between *cdx4* and *foxd3* during the specification of posterior NC cells. In this thesis, I speculate that Cdx4 and Foxd3 may cooperate during NC cell specification based on their binding at shared putative enhancers near NPB genes. However, this claim warrants experimental validation. To test this hypothesis, I propose to count the number of NC cells —identified by anti-Sox10 Antibody immunolabeling — in the posterior-most body region of WT, $cdx4^{ch107-/-}$, Foxd3 MO, and $cdx4^{ch107-/-}$; Foxd3 MO embryos. Moreover, I have shown that Sox10-expressing NC cells do, in fact, form in $cdx4^{ch107-/-}$ mutant embryos, although my preliminary data indicate that the number of NC cells in the posterior-most region of the body is reduced. In *foxd3* mutants, the number of premigratory NC cells remains unaffected, although these embryos express *sox10* and *snai1b* at reduced levels [229, 334]. Further, the formation of cartilage, neuronal, and glial lineages is disrupted in *foxd3* mutants [229, 334], likely due to aberrant apoptosis [334], while pigment cells are not affected [334]. If my hypothesis that Cdx4 and Foxd3 cooperate is correct, I expect that $cdx4^{ch107-/-}$; Foxd3 MO embryos will exhibit a marked reduction, or even complete elimination, of NC cells and their derivatives in the posterior body, as has been reported in double mutant *tfap2a* and *foxd3* embryos [9, 370].

5.2 *cdx4* is necessary for trunk NC cell migration behaviors

The results presented in Chapter 3 indicate that *cdx4* regulates trunk NC migration. Specifically, I demonstrated that $cdx4^{ch107-/-}$ mutants exhibit defects in trunk NC cell migration characterized by failure to form segmental chains and reach ventral positions. My analysis of trunk NC migration *in vivo* revealed that in $cdx4^{ch107-/-}$ mutants, the first NC cell to migrate is regularly overtaken by later cells—an extremely rare event in wild type specimens [287]—and even uncovered instances where two NC cells migrate alongside each other. Moreover, by examining the shape of migrating trunk NC cells, I concluded that *cdx4* is necessary for the establishment of leader cell identity. Together, these results suggest that

cdx4 is necessary for the establishment of trunk-specific behaviors during NC cell migration.

While the role that I propose for *cdx4* in establishing specific NC cellular behaviors might initially appear to lie outside the scope of a NC specifier, its roles parallel those ascribed to the chick cranial NC specifier gene *Ets1*. Ets1 is a component of the chick cranial-specific GRN [325] and there acts to activate the cranial enhancers of *Foxd3* and *Sox10* [23, 326], thus functioning as a *bona fide* NC specifier. In addition, *Ets1* imparts cranial-specific cell behaviors during delamination [347]. Specifically, chick cranial NC cells are capable of delaminating regardless of cell-cycle stage, whereas those in the trunk delaminate during S phase [33, 34, 314, 313]. *Ets1* is necessary for the delamination of cranial NC cells, and its ectopic expression is sufficient to promote cranial-like delamination in trunk NC cells under experimental conditions [347]. In summary, the proposed dual roles of zebrafish *cdx4* in regulating the expression of other early NC specifiers as well as imparting trunk-specific migratory behaviors, are consistent with the largely similar dual roles of chick *Ets1* in the cranial NC.

While the results presented in this thesis suggest that *cdx4* is necessary for establishing leader and follower dynamics, the molecular basis for this distinction and the associated behaviors remains unclear. I propose that in future research, Cdx4 may function as a useful starting point from which to begin elucidating these mechanisms. First, unpublished collaborative studies I performed with the Linker lab suggest that Delta/Notch signaling regulates the establishment of leader/follower identities in premigratory NC cells, such that leader cells are *notch1a*-positive (Alhashem et al. manuscript in prep). Further, our bioinformatics analysis of Cdx4 ChIP-seq data revealed that various genes of the Delta/Notch signaling pathway are bound by Cdx4, including *notch1a*, *notch1b*, and *dla* (data not shown), suggesting that Cdx4 may regulate this pathway. Therefore, I propose that a productive avenue for future experimentation would be to visualize the expression of *notch1a* together with *foxd3* in the progeny of crosses between $cdx4^{ch107+/-}$ heterozygous fish. As our analysis of

cell shape suggests that leader cell formation is impaired in *cdx4*$^{ch107-/-}$ mutants, I predict that the number of *notch1a*-expressing premigratory NC cells, marked by *foxd3* expression, will be reduced or eliminated in these embryos relative to WT or heterozygous siblings.

In addition to their differences in cell shape, it has been proposed that leader and follower cells exhibit differences in the cell cycle stage at which they begin their migration [287]. Additional unpublished results (Alhashem et al., in prep) indicate that leader cells preferentially begin their migration in S/G2/M phase while followers begin in G1. Moreover, these differences appear to lie downstream of Delta/Notch signaling. Therefore, future experiments could analyze the cell cycle stage at the onset of migration in *cdx4*$^{ch107-/-}$ mutant embryos and WT controls. Specifically, I propose use of the available *Tg(sox10:FUCCI)* line, which possesses fluorescent reporters of cell cycle stage, to visualize the cell cycle phase of trunk NC cells during the onset of migration *in vivo* using single plane illumination microscopy. Given the proposed role for *cdx4* in establishing leader identity, I expect that the first cell to migrate in *cdx4*$^{ch107-/-}$ mutant will generally be in G1, as expected of "follower" cells.

5.3 Cdx4 does not regulate chondrogenesis in NC Cells

In this thesis, I also report that *cdx4* is neither necessary not sufficient for repressing chondrogenic potential in trunk NC cells. As shown in Appendix A, I first evaluated the role of Cdx4 in regulating chondrogenesis by global overexpression of *cdx4* at the stages during which cranial NC cells migrate into the pharyngeal arches and begin to differentiate into chondrogenic precursors. In these specimens, the ectopic expression of *cdx4* resulted in loss of expression of the chondrocyte marker *dlx2a* by 24 hpf. Further, these specimens also exhibited defects in the cartilaginous derivatives of cranial NC cells in the pharyngeal basket by 5 dpf. The defects I observed following global overexpression of *cdx4* were consistent with phenotypes previously observed when NC development is abrogated. In fact, *dlx2a* expression in the pharyngeal arches is similarly reduced or eliminated in *tfap2a* mutant em-

bryos [159, 160], and in specimens that have been injected with morpholinos against Tfp2a and Tfap2c [128]. These latter double knock-down specimens exhibited impaired pharyngeal cartilage formation, characterized by either stunted and mis-patterned elements or even complete loss of cartilage [128, 159, 160, 182]. Similar defects in NC-derived cartilage formation also result from the loss of *foxd3* function, either in mutants or following morpholino injections [187, 229, 334]. Therefore, I initially hypothesized that the phenotype observed following global overexpression of *cdx4* was consistent with a role in repressing chondrogenic capacity in NC cells.

However, more focused experimental interrogation (Appendix A) does not corroborate that the observed defect in chondrogenesis reflects an endogenous role for *cdx4* in NC cells. First, loss of Cdx function, in either $cdx4^{ch107-/-}$ mutants or double Cdx1a/4 MO knockdowns, does not lead to posterior expansion of markers of chondrogenic fate, *dlx2a* and *twist1a*. Thus, Cdx function is not necessary for repressing chondrogenic potential in trunk NC cells. Second, in order to build on the preliminary gain of function experiments, I investigated whether expression of *cdx4* specifically in cranial NC cells would inhibit their capacity to form chondrocytes. This approach allowed me to interrogate whether the defects in cartilage development observed following global overexpression of *cdx4* were due to its function in NC cells versus the result of alterations to the signaling environment. To achieve NC-specific expression I used the gateway cloning system to engineer a Tol2 transgenesis construct, in which the -7.2kb regulatory region of *sox10* drives expression of *cdx4*, as well as a *venus* reporter, in NC cells. Strikingly, in embryos injected with this construct and transposase mRNA, the ectopic expression of *cdx4* in cranial NC was not sufficient to inhibit *dlx2a* expression nor to repress differentiation into cartilage. Together, these results are not consistent with my initial hypothesis that *cdx4* represses chondrogenic potential in trunk NC cells.

Given that *cdx4* does not appear to function specifically in NC cells, it follows that the

phenotype observed following global overexpression of *cdx4* is not due to its cell autonomous function in NC cells but rather due to alterations in the migratory environment. In the pharyngeal region, the cranial NC cells surround a mesodermal core and are lined internally by endoderm and externally by ectoderm [232]. Ectopic expression of *cdx4* could impair the development of either of these tissues, which are necessary for the survival, differentiation, patterning, and morphogenesis of the NC-derived craniofacial cartilage [225]. As such, these specimens likely exhibit a defect in the formation of craniofacial cartilage that does not result from a NC cell-autonomous function of *cdx4* in repressing chondrogenic potential, but rather an impaired environment.

It is also possible that the defects in chondrogenesis observed following global overexpression of *cdx4* could have arisen secondarily to impaired heart development. Specifically, the global *cdx4* overexpression specimens had small and thin hearts that did not undergo looping and exhibited significant pericardial effusion by 5 dpf. Surprisingly, $cdx4^{ch107-/-}$ mutants also appear to have pericardial effusion, as well as stunted and mispatterned pharyngeal cartilage by 5 dpf (data not shown). Thus, it is possible that either the pericardial effusion or the impaired heart development abrogate the development of the pharyngeal cartilage.

It is tempting to suggest that the impaired heart development observed in these two conditions could be caused by defects in the cardiac NC cells. In zebrafish, NC cells originating from a region that extends from the midbrain/hindbrain boundary to the sixth somite contribute to the myocardium, as well as the bulbus arterious and the outflow tract of the heart [43, 183, 299]. More recently, NC cells have also been shown to contribute to heart regeneration in adult zebrafish [342]. Notably, laser ablation of NC cells anterior to somite 1 resulted in similar heart development defects, namely impaired looping and pericardial effusion, as early as 48 hpf [183]. However, it is also possible that these defects in heart development arise due to the role of Cdx factors in patterning the anterior lateral plate mesoderm. In both mice and zebrafish, Cdx factors bind near many genes involved in heart development

and hematopoiesis [90, 262], including *Tbx5* and *Nkx2-5* in mice [90]. In zebrafish, interactions between Cdx factors and the retinoic acid signaling pathway—which is itself a target of Cdx—pattern the lateral plate mesoderm and regulate the *tbx5a*-expressing domain that will give rise to cardiac progenitors [178, 281]. Therefore, while it is possible that impaired heart development and pericardial effusion may account for the defect in pharyngeal cartilage formation, it remains unclear whether this is the result of altered cardiac NC cells or mispatterned anterior lateral plate mesoderm.

5.4 Composition and function of the trunk NC GRN

My finding that zebrafish *cdx4* does not act to repress the chondrogenic potential of trunk NC cells is striking in that it highlights our limited understanding of the function of the trunk NC GRN in regulating axial-specific properties. While our understanding of the genetic circuits that orchestrate NC cell development has greatly increased in recent years (see Chapter 1), these studies have largely focused on the cranial NC cell population. In fact, detailed examination of the trunk-specific NC cell GRN has not yet been described and remains an important gap in the field.

Previous studies from chick and mouse embryos have revealed that a cranial-specific genetic circuit mediates the capacity of NC cells to form chondrogenic derivatives [325, 330]. In fact, ectopic expression of components of the cranial GRN is sufficient to allow the formation of cartilage from trunk NC cells [325, 330]. In this thesis, I initially hypothesized that one of the possible roles of Cdx factors in the trunk NC GRN would be to repress the capacity to form cartilage. However, given that my data do not fit this model, it is reasonable to speculate that the battery of genes that orchestrate chondrogenic differentiation represent a cranial-specific elaboration that does not need to be repressed in trunk NC cells.

Such a cranial-specific elaboration is consistent with data reported by Martik and colleagues (2019) [197] suggesting that the cranial GRN evolved via gradual acquisition of the

component genes. Briefly, by visualizing the expression of the genes that make up the chick cranial NC GRN in lamprey, skate, and zebrafish embryos, this study concluded that the components of the cranial circuit were added progressively over evolutionary time. In lamprey embryos, the components of the chick cranial NC GRN are not detected in early NC cells, although they are later expressed in the pharyngeal arches. In zebrafish and skate embryos, *ets1* is detected in both cranial and trunk NC cells. Further, zebrafish cranial NC cells also express *lxh5* and *dmbx1a*, but not *brn3* [197]. Therefore, the components of the cranial NC GRN initially described in chick embryos appear to have been progressively acquired over vertebrate evolution. Notably, while *ets1* is also present in the trunk NC cells in skate and zebrafish embryos, and only later restricted to the cranial region in chicks, the other genes that make up this circuit are only present in cranial NC cells and therefore may not need to be repressed in the trunk.

As evidence for the progressive regionalization of the NC throughout evolution, Martik and colleagues (2019) [197] also found that lamprey cranial NC cells more closely resemble chick trunk NC cells based on hierarchical clustering of transcriptional profiles from cranial and trunk NC cells from these two species. In this thesis, I have performed RNA-seq on zebrafish cranial and trunk NC cells (Appendix B). Therefore, future investigations could integrate the results from my transcriptional profiling of cranial and trunk NC cells from zebrafish embryos, a teleost fish representing an important class of vertebrates, with the hierarchical clustering analysis described above. Briefly, Martik and colleagues generated a "conserved transcriptome" and mapped the reads from their RNA-seq to this dataset. I suggest establishment of a similar "conserved transcriptome" that also includes the zebrafish as a reference. After mapping the reads to this new dataset, hierarchical clustering of all known NC genes could be performed across the three species. I expect that certain groups of genes will be of particular interest, especially axial-specific enriched genes that are conserved in all three species and genes that are enriched in axial populations in two species, but not

the third. By including a teleost fish in the comparison between a jawless vertebrate and an amniote, this analysis may greatly inform our models of how the regionalization of the NC along the body axis emerged over evolutionary time (see [288]). In particular, it may help resolve the apparent conflict between the findings of paleontology and comparative anatomy, which suggest that ectomesenchymal potential is an ancestral condition [328, 329], and those of more recent genomic analyses, which propose that differentiation modules that encode for novel derivatives (including jaws) have been added to the GRN [111].

The results presented in this thesis and in the literature described above highlight the importance of elucidating the genetic circuits that underlies the development of trunk NC cells. I have begun to address this question by profiling the transcriptomes of cranial and trunk NC cells in zebrafish embryos and identifying transcription factors and signaling pathway components that are enriched in trunk NC cells (Appendix B). While this has uncovered candidate genes that may function in the development of trunk NC cells, in the future it will be critical to build on these initial results with additional genomic analyses. In particular, I propose that future approaches ought to reverse engineer the trunk NC GRN as described by Williams et al. (2019) [375] and Ling and Sauka-Spengler (2019) [185] for the cranial and vagal NC populations, respectively, in chick embryos. Briefly, by characterizing the transcriptional signatures and cis-regulatory landscape of these cell populations, they have been able to uncover the logic of the regulatory networks that orchestrate NC cell development. Thus, a similar analysis is possible based on RNA-seq and ATAC-seq of FAC-sorted *foxd3:mCherry* [127] trunk NC cells from zebrafish embryos. Moreover, by performing this analysis in WT and *cdx4*$^{ch107-/-}$ embryos, one could illuminate the role of Cdx4 within the NC GRN, as has been the case with *foxd3* [190], to better understand its function as an early NC cell specifier.

APPENDIX A

CDX FACTORS DO NOT REPRESS CHONDROGENIC POTENTIAL IN TRUNK NC CELLS

A.1 Abstract

One of the most striking manifestations of NC cell regionalization along the AP axis lies in the differences in differentiation potential between cranial and trunk subpopulations. While cranial NC cells give rise to bone and cartilage that make up much of the craniofacial skeleton, trunk NC cells lack the capacity to form these derivatives even when placed in the appropriate signaling environment. As Cdx factors have been shown to pattern the neural plate along the AP axis in zebrafish and mice, I investigated whether they play a similar role in NC cell regionalization and repress the chondrogenic potential of trunk NC cells. Here, I show preliminary results, which suggested that global overexpression of *cdx4* impaired the formation of NC-derived pharyngeal cartilage. However, more focused follow up experiments demonstrated that *cdx4* expression specifically in cranial NC cells is, in fact, compatible with cartilage formation. In addition, I also showed that loss of Cdx4 function alone or in combination with Cdx1a did not result in the posterior expansion of chondrogenic fate markers in trunk NC cells. Together, these findings suggest that *cdx4* is neither necessary not sufficient for repressing chondrogenic potential of trunk NC cells.

Attributions

The HCR experiments included in Figure A.3 were performed by Noor Singh.

A.2 Introduction

The capacity to form ectomesenchymal derivatives—including cartilage, connective tissues, dermis, dermal bone and teeth—has long been one of the most fascinating properties of the neural crest (see Chapter 1). Importantly, it is also one of the most striking examples of NC cell regionalization along the AP axis. NC cells contribute extensively to the craniofacial skeleton [225, 298], as discussed in greater detail in Chapters 1.5.2 and 1.6. Yet, lineage-tracing experiments with the quail-chick chimera system revealed that only NC cells that originate anterior to the level of the 5^{th} somite are capable of giving rise to these ectomesenchymal derivatives [172]. Furthermore, a series of reciprocal transplantation experiments revealed that the potential to form ectomesenchyme represents an intrinsic property of cranial NC cells established early in development [239], as discussed in greater detail in Chapter 1.6 and illustrated in Figure 1.5.

Cdx factors have been shown to pattern the developing embryo along the AP axis (see Chapter 1.11) and promote spinal cord fate while repressing hindbrain identity in both zebrafish and mouse [218, 327]. In zebrafish, loss of Cdx4 function alone, or in combination with Cdx1a by morpholino injection, results in the posterior expansion of the hindbrain [327]. In mouse embryos, Cdx factors orchestrate the chromatin remodeling events necessary for establishing spinal cord fate downstream of Wnt signaling [218]. Further, ectopic expression of *cdx4* in zebrafish embryos impairs segmentation of the hindbrain into rhombomeres, and promotes the anterior expression of spinal cord neuronal markers [327]. Additionally, *in vitro* studies with mouse-derived cells have shown that expression of Cdx factors is sufficient to reprogram hindbrain cells to spinal cord fates [218]. Therefore, I hypothesized Cdx factors might play a similar role in patterning the nascent NC along the anteroposterior (AP) axis, namely promoting trunk NC development while repressing the cranial NC program.

To evaluate this prediction, I investigated whether Cdx factors repress chondrogenic capacity—a hallmark of cranial NC cells—in trunk NC cells. Here, I present preliminary

evidence that initially suggested Cdx4 was sufficient to inhibit cartilage formation when globally overexpressed in zebrafish embryos. However, I have further showed that markers of chondrogenic fate are not detected in the trunk of *cdx4*$^{ch107-/-}$ mutants or embryos injected with morpholinos against Cdx1a and Cdx4 (Cdx1a/4 MOs). Additionally, I found that ectopic expression of *cdx4* specifically in cranial NC cells did not inhibit early chondrogenic markers or preclude cartilage formation. Together, my results do not support an endogenous role for *cdx4* in repressing the chondrogenic potential of NC cells.

A.3 Results and Discussion

A.3.1 Global overexpression of cdx4 impairs cartilage formation

Based on the previously described role in patterning the neural plate, I hypothesized that ectopic expression of *cdx4* in cranial NC cells might reprogram cranial NC cells to a trunk-like identity and thus inhibit their capacity to form chondrocytes. To test this hypothesis, I utilized a transgenic line, *Tg(hsp70:cdx4)* [327], in which *cdx4* can be globally overexpressed under control of a heat shock-inducible promoter, to drive expression at two critical stages of NC development. I then assayed for potential changes in molecular and morphological markers of cranial ectomesenchymal NC derivatives. In 24 hpf WT controls, *dlx2a* is expressed in NC-derived chondrogenic progenitors in the pharyngeal arches, as well as in two stripes within the the forebrain (Figure A.1 A). However, when *cdx4* was globally overexpressed 18 hpf—a stage when NC cells enter the pharyngeal arches and begin to differentiate into chondrogenic progenitors—the 24 hpf expression of *dlx2a* in the pharyngeal arches was eliminated, although it was still detected in the forebrain (Figure A.1 B). These data indicated that the global overexpression of *cdx4* is incompatible with the expression of dlx2, a marker of NC-derived chondrocytes.

In zebrafish, cranial NC cells contribute extensively to the craniofacial skull, including the

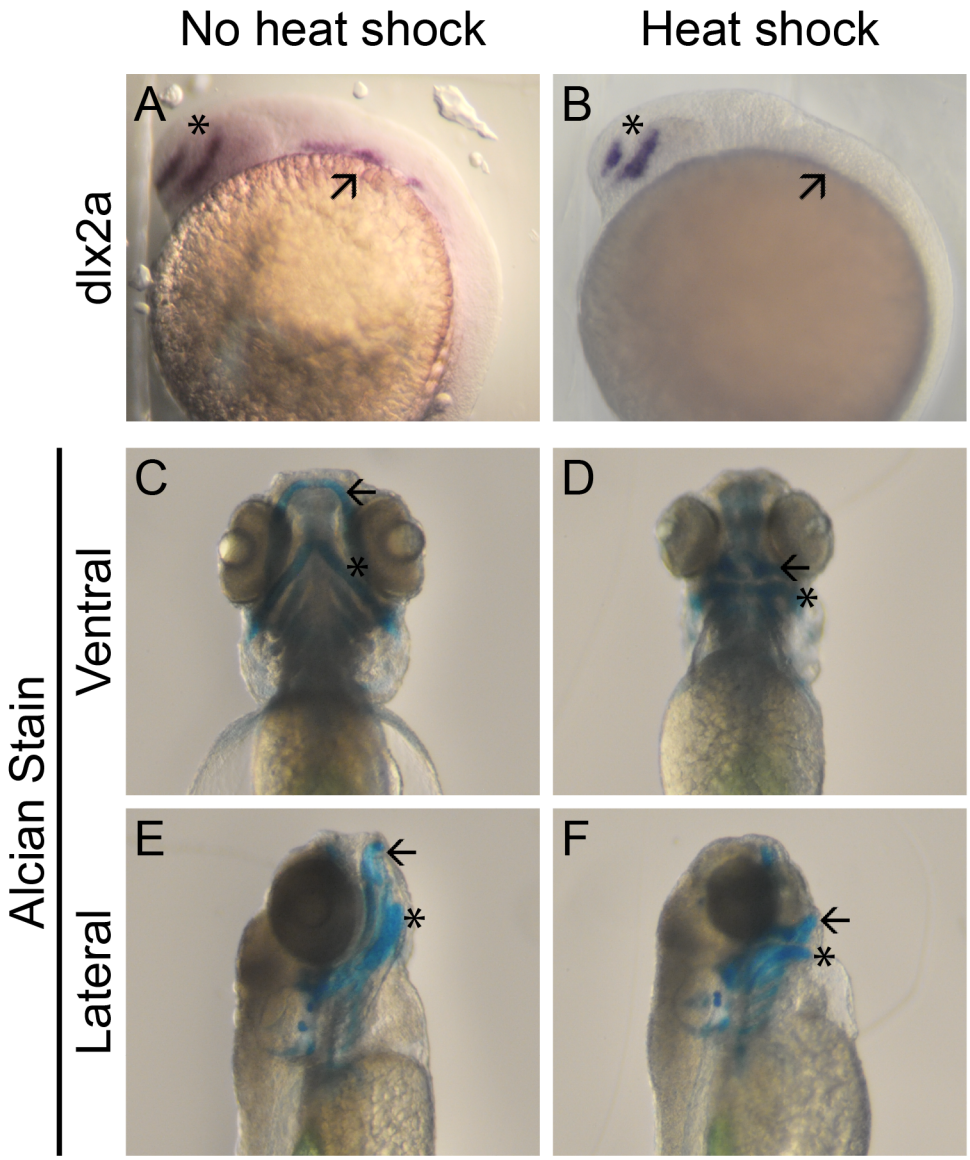

Figure A.1: **Global overexpression of *cdx4* disrupts NC-derived chondrogenesis.** Detection of dlx2a mRNA by in situ reveals (A) expression in the forebrain (*) and pharyngeal arches (arrow) of WT specimens. (B) In heat-shocked embryos *dlx2a* is unaffected in the forebrain (*) but undetectable in the pharyngeal arches (arrow). C, E. Ventral and lateral images of Alcian-stained 5 dpf larvae showing elements of the pharyngeal basket in non-heat shocked controls (arrow = mandibular arch; * = hyoid arch). D, F. Ventral and lateral images of Alcian-stained 5 dpf heat-shocked *Tg(hsp70:cdx4)* larvae showing that the mandibular arch (arrow) and hyoid arch (*) are severely mispatterned and reduced in size.

cartilage of the pharyngeal basket [232]. Therefore, I investigated whether ectopic expression of *cdx4* impaired the formation of NC-derived cartilage. In WT controls, the cartilage of the pharyngeal basket can be labeled with Alcian Green by 5 dpf (Figure A.1 C, E). However, following the global over-expression of *cdx4* between 16-22 hpf, specimens exhibited impaired chondrogenesis, visualized by Alcian staining at 5 dpf (Figure A.1 D, F). Specifically, the NC-derived cartilage elements of the pharyngeal basked appeared reduced in size and mispatterned. Together, these results suggest that expression of *cdx4* during this critical window in cranial NC cell development is incompatible with the formation of NC-derived cartilage.

A.3.2 Cdx4 expression in cranial NC cells does not inhibit chondrogenic differentiation

While the results from the global overexpression of *cdx4* suggested that it may be sufficient to inhibit cartilage formation, this approach also has significant limitations. In particular, because *cdx4* is expressed throughout the embryo using this strategy, I cannot discriminate whether the defects in cartilage formation found in these specimens were due to the cell autonomous function of *cdx4* in cranial NC cells versus non-cell autonomous alterations to the signaling environment.

To distinguish between these two possibilities, I devised an experimental approach to express *cdx4* specifically in NC cells by transient transgenesis. Using multisite gateway cloning, I generated a Tol2 transposon transgenesis construct [164] in which the *sox10 - 7.2 kb* regulatory region—which promotes expression specifically in NC cells—drives the expression of the *cdx4* protein coding sequence. In addition, a *venus* reporter was placed downstream of *cdx4* and joined using a P2A self-cleaving linker [154]. This construct, referred to as *tol2-sox10:cdx4-p2a-venus-tol2*, allowed for ectopic expression of Cdx4 and a Venus fluorescent reporter in NC cells. By injecting the *tol2-sox10:cdx4-p2a-venus-tol2* construct

and transposase mRNA into one cell-stage embryos, I was able to investigate whether the expression of *cdx4* in NC cells was incompatible with chondrogenic differentiation.

To test the hypothesis that *cdx4* is sufficient for inhibiting chondrogenic potential in NC cells, I first determined whether NC-specific *cdx4* expression inhibited *dlx2a* expression. In transient transgenic embryos, I detected the ectopic expression of *cdx4* in NC cells by HCR at 25 hpf (Figure A.2 A). Importantly, however, *dlx2a* transcripts were also detected in these cdx4-expressing cranial NC cells (Figure A.2 B-C). In addition, transient transgenic embryos were allowed to develop to 5 dpf, at which point I analyzed whether *cdx4*-expressing NC cells contributed to the cartilage of the pharyngeal basket. In these specimens, I detected expression of the Venus reporter, which labels *cdx4*-expressing NC cells, in the cartilage of jaw and pharyngeal arches (Figure A.2 D-F, D'-F'). Moreover, these cartilages were patterned in a manner that was indistinguishable from WT controls. Together, these results suggest that *cdx4* expression in NC cells is, in fact, compatible with chondrogenic differentiation.

A.3.3 Cdx function is not necessary for repressing chondrogenic potential in trunk NC cells

Transplantation experiments using the quail-chick chimera system demonstrated that trunk NC cells lack chondrogenic potential even when present in the appropriate signaling environment [239]. In this study, Nakamura and colleagues grafted quail neural primordium from the trunk into the anterior hindbrain of chick hosts. The resulting chimeras lacked facial and branchial skeletal elements [239] indicating that the donor-derived, trunk NC cells were not capable of forming chondrogenic derivatives despite the permissive signaling environment. One possible interpretation of this finding is that the potential to form cartilage is repressed in trunk NC cells. Given that the global overexpression of *cdx4* appears to repress NC-derived cartilage formation, I hypothesized that Cdx factors may function endogenously in trunk NC cells to repress chondrogenic potential. In zebrafish embryos, *cdx4* is expressed

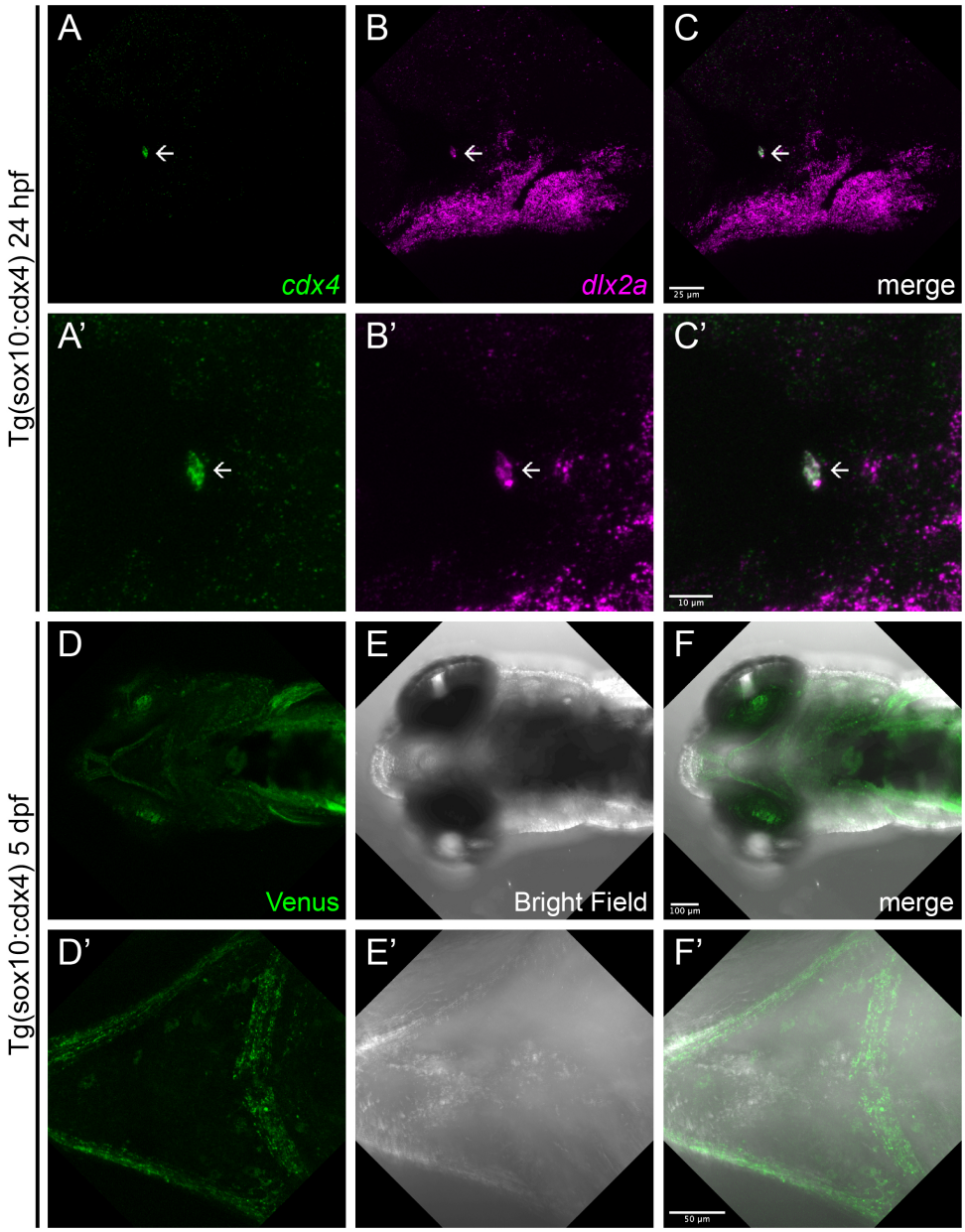

Figure A.2: **Ectopic expression of *cdx4* in cranial NC cells does not inhibit chondrogenic differentiation.** A-B. Images showing the detection of *cdx4* (A) and *dlx2a* (B) mRNA detected by HCR in the cranial region of *Tg(sox10:cdx4)* embryos at 24 hpf. C. Merged image of A and B. Scale bar = 25 µm. A'-C'. Magnified images of A-C. Scale bar = 10 µm. Arrow in A-C and A'-C' labels a cell where *cdx4* and *dlx2a* are co-detected. D-E. Ventral image of Venus expression (A) and bright field (B) in *Tg(sox10:cdx4)* at 5 dpf. F. Merged image of D and E. Scale bar = 100 µm. D'-F'. Magnified images of D'-F'. Scale Bar = 10 µm.

in the tailbud and spinal cord, including the pre-migratory NC cells found at the dorsal neural tube in the trunk (Chapter 3.3.1 and Figure 3.1). Thus, I investigated whether *cdx4* is necessary in trunk NC cells to repress a chondrogenic program.

To test the hypothesis that *cdx4* is necessary for repressing chondrogenic potential in trunk NC cells, I visualized the expression of the chondrogenic markers *twist1a* and *dlx2a* by HCR in the progeny of crosses between $cdx4^{ch107+/-}$ heterozygous fish. In 25 hpf WT and heterozygous siblings, *twist1a* and *dlx2a* transcripts were detected in NC-derived chondrogenic precursors in the pharyngeal arches (Figure A.3 A-D). In the posterior body, *twist1a* was also detected in the posterior paraxial mesoderm, but neither marker was detected in Sox10-expressing NC cells (Figure A.3 E-H). In $cdx4^{ch107-/-}$ mutants, *dlx2a* and *twist1a* transcripts were also detected in the pharyngeal arches. Contrary to my prediction, however, these markers were not detected in Sox10-expressing NC cells in the trunk or tail (Figure A.3 I-L). Therefore, loss of Cdx4 does not appear to result in a posterior expansion of chondrogenic potential.

Previous studies in both zebrafish [45, 65, 66, 327], and mouse [46, 362, 363, 365, 384] have demonstrated that Cdx factors function redundantly in regulating the development of the posterior body. In zebrafish, loss of Cdx1a in addition to Cdx4 markedly exacerbates the truncation phenotype [66]. Further, Cdx1a and Cdx4 function redundantly in the specification of spinal cord identity [327]. Thus, I analyzed whether Cdx1a and Cdx4 function redundantly to repress chondrogenic potential in trunk NC cells by injecting embryos with morpholinos against both of these Cdx factors (Cdx1a/4 MOs) and again visualizing the expression of chondrogenic markers. In 25 hpf Cdx1a/4 MOs, neither *dlx2a* nor *twist1a* transcripts were detected in Sox10-expressing NC cells in the posterior body, while their expression in the pharyngeal arches was unaffected (Figure A.3 M-P). Thus, the loss of Cdx4 alone or in combination with Cdx1a does not lead to the de-repression of chondrogenic markers in the posterior body as predicted by my hypothesis.

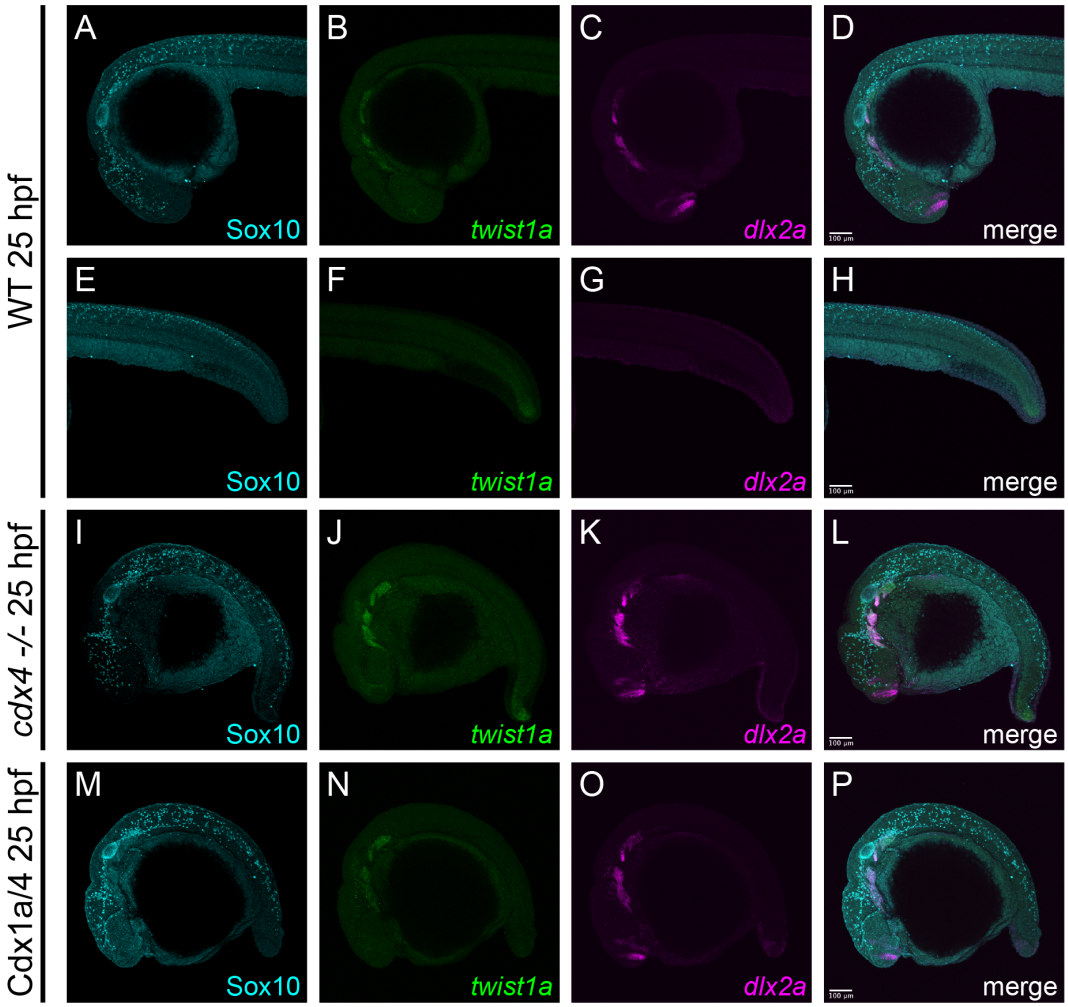

Figure A.3: ***cdx4* is not necessary for repressing chondrogenic potential of trunk NC cells.** Images showing simultaneous detection of Sox10 (A) protein detected by immunohistochemistry, and *twist1a* (B), and *dlx2a* (C) mRNA detected by HCR in the anterior of 25 hpf WT embryos. D. Merged image of A-C. E-G. Images showing simultaneous detection of Sox10 (E), *twist1a* (F), and *dlx2a* (G) in the posterior end of 25 hpf WT embryos. H. Merged image E-G. I-K. Images showing simultaneous detection of Sox10 (I), *twist1a* (J), and *dlx2a* (K) in $cdx4^{ch107-/-}$ mutant embryos at 25 hpf. L. Merged image I-K. M-O. Images showing simultaneous detection of Sox10 (M), *twist1a* (N), and *dlx2a* (O) in the anterior of 25 hpf WT embryos. P. Merged image M-O. Scale bars = 100 µm.

A.4 Materials and Methods

A.4.1 Heat shocks

Expression of *cdx4* in *Tg(hsp70:cdx4)* embryos, was induced by incubating the specimens for 1 hour at 37°C, as described by Skromne [327]. Induction of robust expression was validated by detecting *cdx4* mRNA by *in situ* hybridization.

A.4.2 In situ hybridization

Detection of *cdx4* and *dlx2a* by in situ hybridization was carried out as previously described [279] using NBT/ BCIP as the enzyme substrate. Embryos were cleared in 70% dimethylformamide overnight at 4 °C, washed twice with 100% Methanol for 30 min at room temperature, and then mounted in 80% glycerol in PBS + 0.1%Tween-20. Images were acquired using a Leica DFC490 camera on a Zeiss Axioskop microscope.

A.4.3 Alcian Green Stains

To examine pharyngeal cartilage development, 5 dpf larvae were stained with Alcian green. Specimens were fixed in 4% PFA overnight, washed in 1x PBS + 0.1% Tween-20, and incubated in an ascending series of ethanol concentrations ranging from 30-95%. Larvae were then transferred to acid alcohol (80% ethanol, 20% glacial acetic acid) for 1 hour at room temperature and later incubated in 0.2% Alcian green (in acid alcohol) overnight at room temperature on rocker. The following day, embryos were washed with acid alcohol and a series of ethanol concentrations ranging from 95-30%. These specimens were then washed with dH2O and 3% H2O2, 0.1% KOH solution for 2 hours at room temperature. Finally, the samples were washed with dH2O and stored in 80% glycerol.

A.4.4 Molecular Biology and microinjections

The *tol2-sox10:cdx4-p2a-venus-tol2* was cloned using a standard multisite Gateway (L-R) reactions, as described [164]. The *pDestTol2CG2* and *p3E-polyA* were obtained from the Tol2kit v1.2 [164]. The *p5E-sox10(-7.2kb)* plasmid was a kind gift of Bruce Appel and the *pME-cdx4* was obtained from Twist Biosciences (San Francisco, CA). The *tol2-sox10:cdx4-p2a-venus-tol2* construct was injected as circular DNA into single-cell *AB embryos at a concentration of 80 ng/ µl, together with capped Tol2 transposase mRNA—transcribed using the MEGAscript SP6 Kit (Ambion) according to manufacturer's instructions—at a concentration of 80 ng/ µl, as described [18].

APPENDIX B
TRANSCRIPTIONAL PROFILING OF CRANIAL AND TRUNK NC CELLS IN ZEBRAFISH EMBRYOS

B.1 Abstract

The development of NC cells is orchestrated by a gene regulatory network that consists of a series of interlinked modules. In recent years, transcriptional profiling of cranial and trunk NC cells has revealed that axial-specific genetic circuits underlie the unique properties of these subpopulations. In collaboration with Megan Martik and Marianne Bronner (CalTech), I investigated the differences in gene expression between zebrafish cranial and trunk NC cells and identified a set of candidate genes that may pattern the NC along the anteroposterior axis.

B.2 Introduction

NC cell development is a complex, multi-step process consisting of induction and specification at the neural plate border (NPB), an epithelial-to-mesenchymal transition and subsequent migration, and finally differentiation into its various derivatives (see Chapter 1). Further, NC cell development is regulated by cell-extrinsic signals and cell-intrinsic gene regulatory interactions, which can be organized into a gene regulatory network (GRN) [324]. This GRN, discussed in Chapter 1 and illustrated in Figure 1.3, consists of a series of interlinked modules that orchestrate the various steps of NC cell development.

It has been proposed that differences in the GRNs that operate within NC cell subpopulations along the AP axis may underlie their distinct properties, including their differentiation capacity [324]. In recent years, a wealth of genomic approaches, described in detail in Chapter 1.7, have provided evidence to support this assertion. Notably, differences in

gene expression between cranial and trunk NC cells appear to regulate their developmental potential in chick and mouse embryos [325, 330]. In chick embryos, a cranial-specific GRN that functions during NC induction, specification, and early migration underlies the capacity of cranial NC cells to form chondrocytes [325]. In mouse embryos, sustained expression of the *Twist1* gene in the cranial region biases NC cells towards an ectomesenchymal fate [330]. In both species, ectopic expression of components of the cranial GRN in trunk NC cells is sufficient for promoting chondrogenic differentiation.

In this thesis, I aimed to build on these reports by interrogating the gene-regulatory basis of cranial and trunk NC cell identity in zebrafish embryos. Here, I describe experiments in which I performed transcriptional profiling of FAC-sorted cranial and trunk zebrafish NC Cells. This approach uncovered a set of candidate genes that may regulate the axial-specific properties of cranial and trunk NC cells in zebrafish. Further, this dataset can serve as a foundation for future experiments aimed at uncovering the gene-regulatory mechanisms by which the NC is patterned along the AP axis, as discussed in Chapter 4.

B.3 Results and Discussion

In recent years, there has been a growing body of evidence that NC cell regionalization is regulated by axial-specific GRNs [185, 375], and that distinct gene expression programs mediate unique properties of distinct subpopulations of NC cells along the AP axis [100, 325, 330]. To identify the molecular mechanisms that govern the unique properties of cranial and trunk NC Cells in zebrafish embryos, I visited Dr. Marianne Bronner's lab at CalTech, and worked together with Dr. Megan Martik to profile the transcriptomes of these subpopulations. To this end, we used the *gt(foxd3:mCherry)* transgenic line, a gene trap line that recapitulates endogenous expression of *foxd3* and labels premigratory NC cells [127]. We dissected transgenic embryos to isolate the head tissue anterior to the 1st somite at 11 hpf, and trunk tissue between the 7th somite and the tailbud at 14 hpf, which enabled the isolation of cranial and

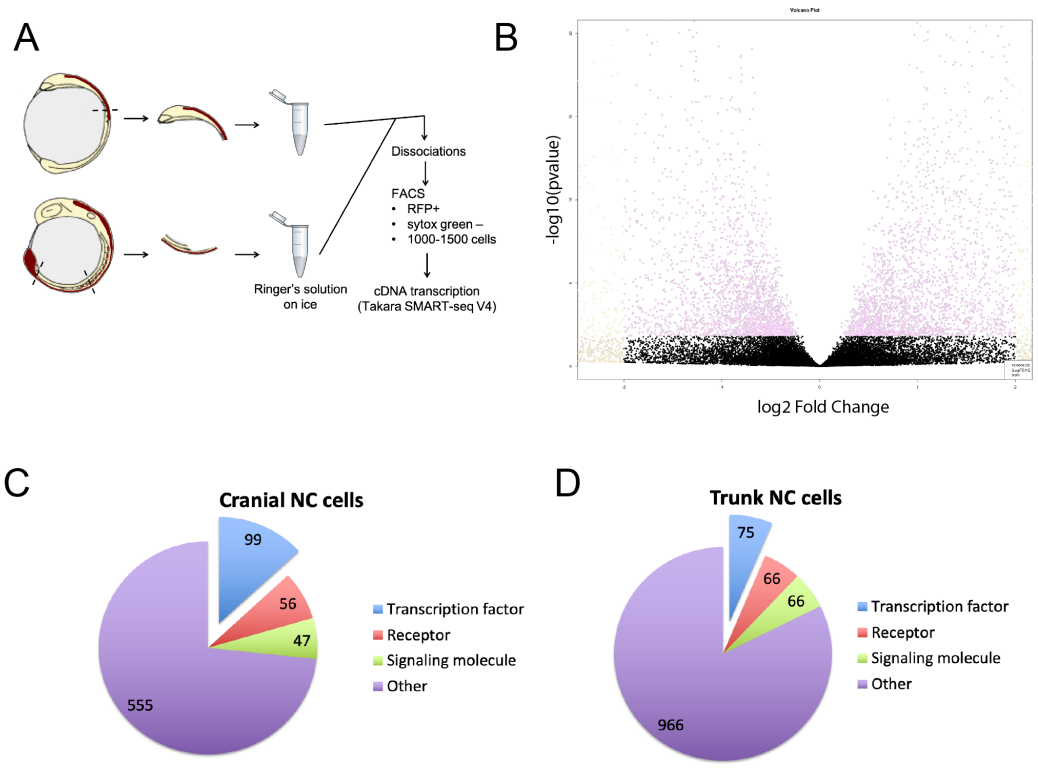

Figure B.1: **Transcriptional profiling of cranial and trunk NC cells.** A. Schematic of experimental approach for isolating cranial and trunk NC cells from zebrafish embryos and performing RNA-sequencing on these axial populations. B. Volcano plot showing differential gene expression between cranial and trunk NC cells. C. Representation of Gene Ontology analysis of cranial-enriched genes, highlighting transcription factors, signaling molecules, and receptors. D. Representation of Gene Ontology analysis of trunk-enriched genes, highlighting transcription factors, signaling molecules, and receptors.

trunk NC cell populations, respectively, by fluorescence-activated cell sorting (FACS) (Figure B.1 A). Following library preparation, sequencing, and differential expression analysis, we identified 920 and 1421 genes enriched in cranial NC cells and trunk NC cells, respectively (Figure B.1 B).

To identify candidate genes that may play a role in patterning the NC along the AP axis, I performed Gene Ontology analysis, focusing on transcription factors, signaling molecules, and receptors. This analysis identified 99 transcription factors, 47 signaling molecules, and 55 receptors enriched in the cranial NC (Figure B.1 C). Similarly, 75 transcription factors, 66

signaling molecules, and 66 receptors were enriched in the trunk NC (Figure B.1 D). Next, I examined the spatial expression patterns of these genes using data available on zfin.org, an online database for zebrafish genetic data. From these data, I selected genes that were expressed in NC cells or in the developing neural tubes in a spatially-restricted manner along the AP axis. From this analysis, I identified 38 genes and 15 genes enriched in cranial and trunk NC cells, respectively (Figure B.2). These genes represent promising candidates that may play a role in establishing cranial or trunk NC identity. Importantly, orthologs of known cranial NC genes from chick and mouse, including *dmbx1a/b*, *lmx1bb*, and *twist1a* were enriched in the cranial population. As further proof of principle, *cdx4* and *cdx1a* were highly enriched in trunk NC cells.

B.4 Materials and Methods

B.4.1 RNA sequencing and analysis

Head and trunk tissues were dissected from gt(foxd3:mCherry) emrbyos and placed in Ringer's solution on ice. mCherry-expressing NC cells were isolated by FAC-sorting (BD Biosciences FACSAriaFusion) and pooled into samples containing 1500 cells. cDNA was then transcribed using the SMART-seq Ultra Low Input RNA Kit V4 (Takara). Sequencing libraries were then prepared and sequenced at the Millard and Muriel Jacobs Genetics and Genomics Laboratory at CalTech (Illumina HiSeq2500). Reads were then mapped to the zebrafish genome (GRCz10) using Bowtie2. Transcript counts were calculated with featureCounts, and genes that were differentially expressed were determined using DESeq2.

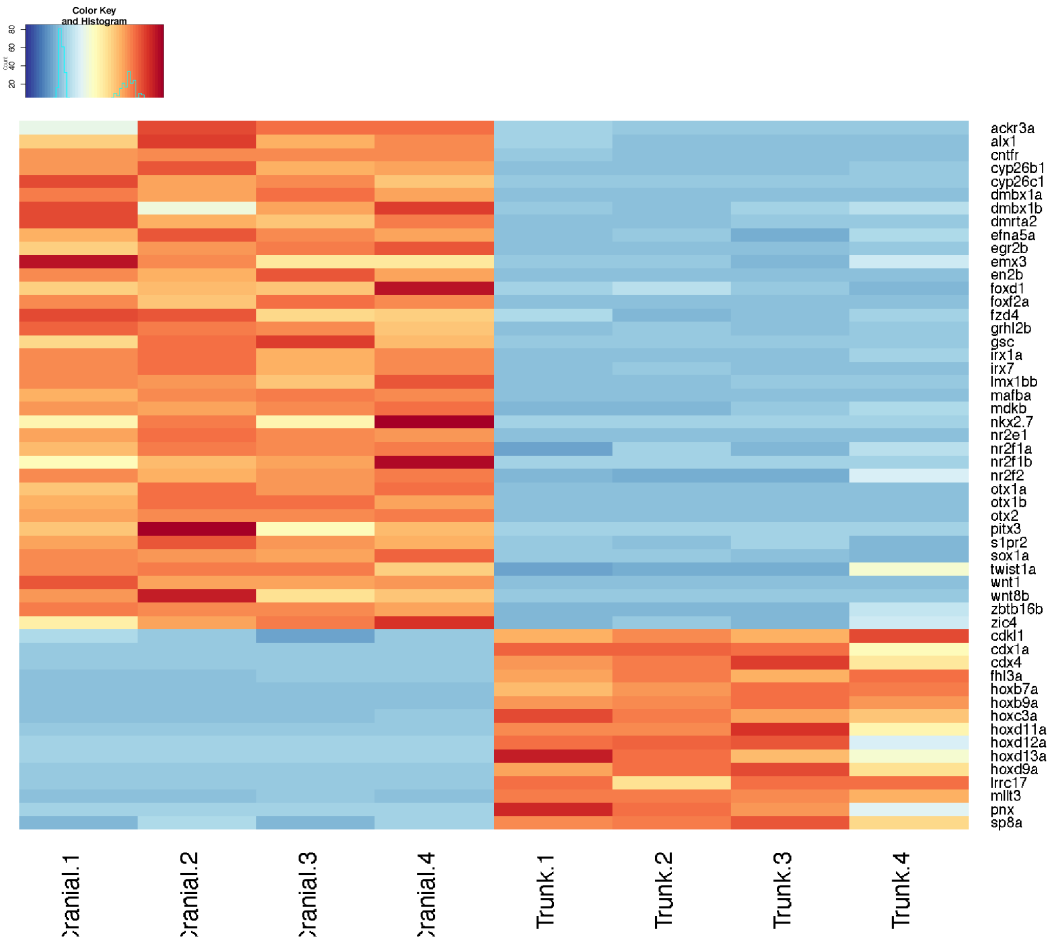

Figure B.2: **Candidate genes enriched in cranial and trunk NC cells.** Heat map of candidate genes that are enriched in cranial or trunk NC cells. Notably, the expression of these genes in the neural plate and/or NC cells was validated based on publically-available expression data on zfin.org.

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