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Cellular and Molecular Mechanisms of Collective Migration in Facial Branchiomotor Neurons

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CELLULAR AND MOLECULAR MECHANISMS OF COLLECTIVE NEURON MIGRATION IN FACIAL BRANCHIOMOTOR NEURONS

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Abstract

Cellular and Molecular Mechanisms of collective migration in Facial Branchiomotor Neurons

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The directed migration of neurons is influenced by multiple guidance cues, that may include soluble attractive chemotactic factors and cell-cell contact mediated collective migration. The nature of these neuron-neuron interactions and their integration with chemotaxis remains unclear. Contact inhibition of locomotion (CIL), a process whereby cells undergoing a collision cease their migration towards the colliding cell, has been identified as a driving force behind the collective migration of several cell populations *in vivo*, but has not been described for neurons in the central nervous system. We have established that Cadherin2 (Cdh2), a cell adhesion molecule, mediates the physical interactions between facial branchiomotor neurons (FBMNs) that promote the collective mode of migration. Using live imaging, we observed transient cell-cell contact between the somas of FBMNs during migration. Following neuron-neuron collisions, we observed two directional outcomes: i) both neurons remain travelling posteriorly, or ii) the neurons migrate in opposite directions (one anterior and one posterior). This latter observation is a hallmark of CIL behavior. These CIL events occur in approximately 50% of soma-soma collisions. Consistent with the repulsive nature of CIL events, live imaging of *Tg(isl1:GFP-CAAX)* fish show that CIL events are characterized by a collapse of protrusions upon collision. Our data indicate that CIL-based neuron-neuron interactions influence the directionality of FBMN movement and may underlie the collective nature of FBMN migration. To determine whether chemotaxis could influence FBMN directionality after cell-cell collisions, we examined the interplay between Cdh2-mediated collective migration and SDF1a-mediated chemotaxis. We found partial FBMN migration defects under conditions when Cdh2 function is partially inactivated or when the chemokine SDF1a is knocked down. Strikingly, we find an almost complete migration block when both SDF1a is depleted and Cdh2 function is inactivated. These findings suggest that FBMNs integrate multiple inputs arising from cell-cell contact induced polarity changes and SDF1a-mediated chemotaxis to achieve sustained directed migration.

TABLE OF CONTENTS

List of Figures

Chapter 1: Introduction

Fundamental Steps in Neural Development

Neural development in vertebrates involves several key steps to produce a functioning nervous system: neural induction, neurogenesis and proliferation, cell migration, circuit formation and process outgrowth, synaptogenesis, neuronal death and synaptic rearrangement. Each step is a highly organized process, and not surprisingly, involves many cellular and molecular mechanisms. It is well established that mutations in genes involved in any of these steps result in defects in overall brain size and organization, connectivity, a range of neurodevelopmental disorders and developmental delays (Ross and Walsh, 2001).

Neurogenesis and proliferation begins with the process of neural induction. During this essential period, ectodermal cells are set aside to become the neural plate by signals from mesodermal cells. Mesodermal cells secrete Noggin and Chordin to inhibit BMP-signaling required for epidermal cells to differentiate into the epidermis. This inhibition of BMP signaling by Noggin and Chordin is both necessary and sufficient for differentiation of the ectoderm into neural tissue and formation of the neural tube (Ezin et al., 2009; Kishimoto et al., 1997; Wallingford, 2006). Once neural tissue has formed, regional segmentation occurs in the nervous system along both the anterior-posterior and dorsal-ventral axis of the developing neural tube. There is a graded concentration of diffusible signals restricting the expression of specific transcription factors, which act on downstream targets to allow regional identity in the nervous system (Kishimoto et al., 1997).

Neurogenesis also encompasses proliferation of cells in the neural tube. Initially, the neural tube is only a single cell layer thick. Progenitors of the nervous system, neural stem cells (NSCs), at this stage begin undergoing rapid mitotic divisions to increase their cell population. Importantly, many NSCs at this stage have a bipolar morphology with one process

extending to the middle of the neural tube (ventricular zone) and the other to the outer pial surface. These elongated NSCs are referred to as radial glial progenitor cells. Radial glial progenitor cells produce both neurons and glial cells in the central nervous system. Radial glial cell nuclei move from the inside to the outside the neural tube during each cell cycle, in a process termed interkinetic nuclear migration. Inner nuclear movements prompt cell division into two daughter cells, which quickly migrate outward again. These divisions produce either new NSCs or neuroblasts that eventually become neurons. Earlier cell divisions are symmetric and produce two new NSCs whereas later divisions are asymmetric to produce one NSC and one post-mitotic cell. Post-mitotic cells no longer divide and become specialized cells of the nervous system (Beattie and Hippenmeyer, 2017).

After neurogenesis is complete, newly born neurons must migrate to their fated destinations. Neuronal migration (see below) is a highly regulated, directed movement of cells regulated by the expression of several molecules (details discussed further later). Neuron migration can occur radially or tangentially, but both are necessary for the developing nervous system (for more information, see below) (Meglio and Rijli, 2013; Nadarajah et al., 2001).

After neuron migration, axon and dendrite process outgrowth occurs. In order for the nervous system to establish and maintain functional circuits, axons must navigate through tissues to find proper targets prior to forming synapses. Additionally, dendrites also grow extensively from the neuron cell body, which will receive synaptic inputs. A neuron's axon often navigates long distances, responding to multiple guidance cues to reach its target. To achieve this, axons express guidance receptors on their growth cone, which respond to specific secreted ligands and also respond to contact-mediated cues. These cues may be attractive or repulsive, as both are required for proper path finding. Both secreted and contact-mediated cues allow axons to integrate cues to ensure proper directional choices (Tessier-Lavigne, Marc; Goodman, 1996).

As stated above, dendrites are important regulators of neuronal circuits, as they receive synaptic input information. Dendrite outgrowth varies widely depending upon the type of neuron and allows us to distinguish morphologically between types of neurons. Dendritic arborization is more complex than axon path finding and we know less about the molecular signals that control dendritic arbor size and morphology. A neuron can have several dendrites receiving input signals and the structure of these dendrites has a critical impact on processing neuronal information. Most studies exploring cellular and molecular mechanisms of dendritic outgrowth are conducted on pyramidal neurons of the cortex, because of their characteristic dendritic outgrowths. They have a prominent apical dendrite and multiple basal dendrites, thus giving a pyramid shape. Regardless of the type of neuron, dendrites must grow properly to relay information and maintain neuronal circuits. Thus, defects in the cellular and molecular mechanisms of dendritic outgrowth can have severe pathological consequences. Defects in dendritic arborization have been associated with patients suffering from problems with learning and memory as well as autism, schizophrenia and Alzheimer's Disease (Arikkath et al., 2012).

Once process outgrowth is complete, synaptogenesis occurs. These functional connections distinguish brain morphogenesis from that of other organs in vertebrate development. Synaptogenesis, in its simplest terms, is the formation of cell-cell connections allowing electrical or chemical signaling between two synaptic partners (presynaptic neuron and postsynaptic target). Accumulating evidence has implicated binding of some form of cell adhesion molecule as essential in the formation of synapses. Binding of these CAMs leads to recruitment of synaptic components on both pre-synaptic and post-synaptic cells. Examples of these cell adhesion molecules include Cadherin-2, DSCam, Syn-Cam, Neurexin-neuroligin, to name a few. Although the scope of this thesis does not specifically investigate molecular modulators of synaptogenesis, it is important to note that mutations in these modifier genes can have severe implications on learning, memory and cognition in the adult brain (Waites et

al., 2005).

The final step in neural development is cell death and synapse rearrangement. Neuronal cell death is essential in functional circuit formation. During development, neurons are over-produced and a programmed “die-off” occurs; this programmed cell death of neurons can be triggered in different ways. Firstly, neurons with proper afferent and efferent connections are stabilized, whereas those with no connections or incorrect connections are pruned. Additionally, neurons express specific receptors for specific growth factors called neurotrophins (eg. nerve growth factor (NGF)) secreted by neuronal targets. Those neurons that do not sequester enough NGF ligand will not survive. This is a mechanism that matches the size of the target with the correct innervation density. Lastly, electrical activity in neurons and their target cells helps to maintain or prune neuronal connections (Hutchins and Barger, 1998). This process eliminates weak connections and selects for strong associative connections, and therefore strong synapses. This allows for proper functioning and wiring of the mature brain after development has completed.

The Role of Neuronal Migration in Neural Development

Neuronal migration is an essential step in the development of the central nervous system (CNS) in vertebrate organisms. This process ensures proper wiring of neural circuitry and ultimately organismal behavior. Often, neurons migrate considerable distances from their birthplace to their final destination where they carry out their designated functions. Defects in neuronal migrations result in a variety of neurodevelopmental disorders such as schizophrenia, bipolar disorder, epilepsy, and lissencephaly (Benes and Berretta, 2001; Evsyukova et al., 2013; Ross and Walsh, 2001). Our ultimate goal is to identify the genes responsible for neuronal migration for better pre-screening of patients with potential neurodevelopmental

disorders and to devise novel therapeutic approaches to ameliorate the condition of children born with neurodevelopmental disorders.

During neural development, there are two routes that neurons typically migrate; radially or tangentially. Radial migration occurs when neurons use radial glial cells as scaffolds to migrate from the ventricular zone to the superficial layers in the developing neural tube (Evsyukova et al., 2013). Radial migration has been best studied in the developing mammalian cortex. The cell somas of radial glial cells are located in the ventricular zone and they extend long thin processes through the neural tube to the pial surface, where they are anchored (Valiente and Marín, 2010). Radial glial cells, the stem cells in the developing CNS, undergo asymmetric cell division to produce a new neuron that subsequently attaches to the radial glial parent cell, and migrates radially along its long thin process to the superficial layer of the cortex. Experimental imaging has revealed that neurons that migrate in this manner make intimate associations with the radial glial process, displaying an elongated morphology with long leading process and thin trailing process, appearing to wrap around the glial fiber as it moves from deep to superficial layers, detaching from the radial glial process upon arriving at the outermost layer (Nadarajah et al., 2001). This would suggest that cell adhesion molecules mediate the attachment of newly born neurons to radial glial scaffolds and mediate migration along these scaffolds. Indeed, Cadherin-2, a calcium-dependent homotypic cell adhesion molecule, has been shown to play a role in this process. Inactivation of Cadherin-2 specifically in neurons, but not radial glial cells, impairs radial migration of cortical neurons, with aberrant detachment of neurons from radial glial fibers (Martinez-Garay et al., 2016).

Defects in radial migration, caused by deleterious mutations, can lead to severe neurodevelopmental disorders. For example, mutations in Doublecortin (*Dcx*), causes X-linked lissencephaly, in which patients present with a “smooth” brain malformation causing significant developmental delays such as (Valiente and Marín, 2010). DCX is a microtubule-associated

protein (MAP) that interacts with polymerized microtubules and functions as part of a protein complex to ensure proper migration in the developing brain (Rubenstein and Marín, 2003).

Tangential migration, on the other hand, is characterized by neurons that migrate within the plane of the neuroepithelium, orthogonal to the radial glial cells. That is, tangentially migrating neurons do not require radial glial fibers to guide them to their destination. However, little is known about the substrates that are used by migrating neurons to reach their destination, but presumably include interactions with neural progenitor cells (heterotypic cell interactions), other neurons (homotypic cell interactions), and extracellular matrix proteins. Tangential migration is a highly directional process that requires responsiveness to factors that guide neurons through appropriate pathways to their targets. There are several guidance cues, both attractive and repulsive, that have been implicated with guiding neurons to their proper destination (Guan and Rao, 2003). For instance, the olfactory system relies on the Slit/Robo ligand/receptor signaling as a chemorepellent for proper migration of interneurons to the olfactory bulb (Wu et al., 1999). Another example includes Netrin, a secreted ligand, can act as both a repulsive and attractive cue, depending upon the cell type and receptor present. During development, the receptor for Netrin, UNC-5, attracts axons to the floor plate to ensure proper growth. Alternatively, during development of the embryonic eye, retinal ganglionic cells are guided in their migration by Netrin binding to its receptor Deleted in Colorectal Cancer (DCC) (Kennedy, 2000).

One of the most well studied examples of tangential migration is that of inhibitory interneurons that navigate from the ganglionic eminences to the cerebral cortex (Evsyukova et al., 2013). More specifically, cells born in the medial ganglionic eminence migrate tangentially into the cortex and differentiate into GABAergic interneurons. Multiple guidance cues allow these interneurons to migrate on an extremely specialized path, where they specifically avoid certain areas in the developing parenchyma. These molecules include, but are not limited to,

Slit1/2, Neuropililn1/2 and Sema3A/3F (Rubenstein and Marín, 2003). Mutations in any of these guidance cues can cause migration defects, leading to epilepsy, autism and developmental delays in humans (Fu et al., 2011). For example, interneurons migrating to the cortex express neuropilin1 and neuropilin2 transmembrane receptors, which cause repulsion when activated by their ligands, class 3 semaphorins(Fu et al., 2011; Rubenstein and Marín, 2003). Interneurons destined to invade the striatum do not express neuropilin receptors, thus they cannot respond to semaphorins secreted by the striatum. Additionally, interneurons destined to the cortex bind semaphorins and avoid the striatum to reach their proper cortical target. Consistent with this, loss-of-function mutants in neuropilin1 and neuropilin2 increases the number of interneurons present in the striatum and decreases the number of interneurons reaching the embryonic cortex (Rubenstein and Marín, 2003). Thus, radial and tangential migrations are both required during development to achieve proper CNS patterning and functioning.

General Cell Migration: A Brief Overview

Cell migration is an essential process to the development, functioning, and maintenance of all multicellular organisms. Cell migration in its simplest terms, is the organized movement of cells in specific directions to their proper targets. Particularly, during embryonic development, cells must achieve major re-arrangements to achieve proper tissue and organ morphogenesis. For example, gastrulation in early embryonic development requires large numbers of cells to migrate together to form the three embryonic cell layers: ectoderm, mesoderm and endoderm (Ridley, 2003). Cell migration is also critical in many pathological processes, such as in wound healing where epithelial sheets move en-masse to close a tear, and in the immune response, where individual leukocytes migrate into affected tissues.

Many cells have an intrinsic polarity that provides a front-rear axis to the cell as they

begin to move. This includes the positioning of the nucleus, Golgi, microtubule-organizing center (MTOC) and the arrangement of microtubules to the leading edge. Moreover, a cell with intrinsic polarity may migrate in random directions until guided by a multitude of extrinsic factors. These may include including cell-cell interactions, cell-matrix interactions. Some cells navigate by following a chemo attractive, or chemo repellant gradient of secreted molecules by their target organ, or intermediate guidepost cells(Haastert and Devreotes, 2004). These secreted guidance molecules function by initiating or stabilizing protrusions toward (or away) from the guidance cue, thereby re-orienting the front-rear axis of the cell. Regardless, cell migration requires cells to be polarized with protrusions at the leading edge, leading to assembly of different protein complexes at the front of the cell compared to the rear edge (Carmona-Fontaine et al., 2008). Further mechanisms of cell migration will be discussed later.

Collective Cell Migration

Collective cell migration is defined as the coordinated migration of a cell population through cell-cell cooperation. Previously, collective cell migration was thought to only describe the displacement of a group of cells that remain connected during their movement. For instance, during collective migration of epithelial sheets as observed in wound healing, cells are physically tethered together by stable cell-cell contacts. Other examples include germ layer morphogenesis during gastrulation, blood vessel sprouting, border cell migration in Drosophila, and migration of the lateral line primordium in zebrafish (Scarpa and Mayor, 2016). However, cells can migrate as groups or as individuals, such as in streams. Interestingly, experiments exploring directional migration of neural crest cells suggest collective cell migration can also be mediated by transient cell-cell contacts. These dynamic cell-cell interactions between neighboring cells allow single cells to influence each other and promote coordinated directional movement (Theveneau and Mayor, 2010; Theveneau and Mayor, 2012b; Theveneau and

Mayor, 2013) In conclusion, collective cell migration behavior can occur both in groups of cells (sheets, streams, or clusters), and in individually migrating cells. It is important to review how each of these types of collective migration occurs.

First, we shall summarize collective cell migration in groups of cells physically connected to each other by stable interactions, such as epithelial cells. Cells utilize collective migration through stable cell-cell interactions in different ways in different tissues and organisms. For example, border cells of the *Drosophila* egg chamber or the zebrafish lateral line (pLL) primordium migrate as a small group (Montell, 2003; Theveneau and Mayor, 2012b). In these situations, the cells on the outer edge of the group are polarized and able to physically pull other cells along a migration path. Cells located in the interior of the group are tethered (via cell adhesion molecules) to those around them, enabling them to be pulled but also preventing them from making protrusions (Weber et al., 2012). During general vertebrate wound healing and dorsal closure of *Drosophila* embryos, cells move as a wide sheet (Theveneau and Mayor, 2013). Cell sheet migration in wound healing can be demonstrated in 2D by using a mechanical scratch assay *in vitro* (Trepat et al., 2012). Cells near the edge of the injury (or induced scratch) immediately reorient their centrosomes and Golgi apparatuses to acquire polarity, make membrane protrusions into the free space, and begin to migrate to fill the free space. Similar to small cell groups described above, these polarized cells pull those cells located internally, as they are physically tethered by stable cell-cell contacts (Rørth, 2009). Epithelial cells can also form strands such as those observed in invasive carcinomas. In this case, those at the leading edge are ‘pro-migratory’ and promote tumor invasion into tissues (Deisboeck and Couzin, 2009). Lastly, they can form hollow tubes of epithelial cells such as in blood vessel sprouting. When sprouting occurs, cells at the tip are highly motile with dynamic changes in both lamellopodial and filopodial extensions. At the same time, they also maintain junctions to stalk cells (those not at the tip) to ensure vessel integrity (Wacker and

Gerhardt, 2011). Taken together, cells can migrate as groups, sheets, strands or tubes, but all require front-rear polarity at the 'free' or 'leading' cell edge, as well as physical cellular junctions to the rest of the cell population for proper collective migration.

Alternatively, collective cell migration can also occur between single cells via transient cell interactions, unlike those described above. Single cell collective migration is an important migration mechanism established by *in vivo* studies in neural crest cells, Cajal-Retzius cells and *Drosophila* hemocytes (Davis et al., 2012; Roycroft and Mayor, 2015). Dorsal neural tube cells undergoing epithelial-mesenchymal transition (EMT) to become migratory neural crest cells following neurulation are a common example of single cell collective migration. EMT is a process by which epithelial cells lose stable cell-cell adhesions and apical-basal polarity and become migratory neural crest cells. Although these cells do not maintain stable cell-cell junctions, they do continue to express cell adhesion molecules (CAMs). CAMs assist in transient interactions between cells (Theveneau and Mayor, 2013). Moreover, it has been shown that transient interactions between neural crest cells are essential for their directional collective migration. Without these transient interactions, neural crest cells do not participate in contact inhibition of locomotion, causing them to become invasive, much like malignant cells. These transient interactions are required for proper neural crest cell dispersal to ensure proper tissue growth during embryogenesis (Carmona-Fontaine et al., 2008; Scarpa and Mayor, 2016). Cajal-Retzius (CR) cells are another example of single cell collective migration. These cells are born in the pallium and migrate over the surface of the cortex in order to ensure proper radial migration of neurons later in development. CR cells transiently interact with each other to ensure proper distribution to cover the cortex (Villar-Cerviño et al., 2013). Drosophila *hemocytes* also use collective cell migration during development. Transient cell contacts allow hemocytes to disperse throughout the developing embryo to ensure proper immune response later in life (Davis et al., 2012). Regardless of the cell type, the outcome of

transient cell-cell interactions is a phenomenon called contact inhibition of locomotion (CIL).

Contact Inhibition of Locomotion (CIL): A brief overview

Directed cell migration is required for proper development and functioning of multi-cellular organisms. There are several cellular mechanisms contributing to directed cell migration in multi-cellular organisms. Critically important is the process of Contact inhibition of locomotion (CIL), which mediates proper directional cell migration through physical interactions. Simply put, CIL events suppress forward motion upon cell-cell contact, cause protrusion collapse and result in directionality change away from the direction of the collision. Not only is CIL important during cell migration in developing organisms, but it also plays a role in wound healing and pathological processes like cancer metastasis. Thus, there is an extensive collection of literature on the mechanisms regulating CIL, both *in vivo* and *in vitro*, during development and disease (for reviews, see refs;(Mayor and Carmona-Fontaine, 2010; Roycroft and Mayor, 2016; Stramer and Mayor, 2016). CIL as a phenomenon of cell motility was first investigated and by cell biologist Michael Abercrombie and Joan Heaysman in 1954 when they began studying the “social behavior” of cells. Abercrombie and Heaysman concluded cells are social organisms because tissue culture is a colony of cells, and therefore sought to characterize the social behavior of cells with regard to each other (Mayor and Carmona-Fontaine, 2010). In order to study these phenomena they used tissue explants from the heart of embryonic chicks. They found when migrating cells contacted each other, they 1) stopped migration or dispersed from each other and also 2) a cell would adhere to a substrate and not its neighboring cell. It was determined that free migration of fibroblasts was limited when cells came in contact with other cells, resulting in a decreased velocity inversely proportional to the amount of contacts made with other cells, thus the greater the contacts the greater the decrease in velocity (Roycroft and Mayor, 2015). Since those initial studies, CIL

has been studied in several different systems and cell types and has been established as an important mechanism regulating directed cell migration.

CIL in development, wound healing and cancer dynamics

CIL during development, specifically in mesoderm and neural crest cells

During development, collective migration is essential for proper morphogenesis. Many cells must migrate together in a cohort or group to reach their final destination. Mesendoderm cells are a useful model for studying the role of CIL during collective migration *in vivo*. During gastrulation, mesendoderm cells, termed precordial plate cells, migrate from the embryonic organizer to the animal pole and later give rise to the hatching gland (Dumortier et al., 2012a). Several experiments were conducted to determine if this population of cells exhibits collective migration and how that migration occurs.

Because collective migration can occur differently in different cell populations, experimenters first sought to determine how prechordal plate cells migrate. Using fluorescent markers and 3D imaging techniques, Dumortier and colleagues determined mesendodermal cells move coherently towards the animal pole, all exhibiting similar migrating behaviors of speed, orientation, persistence and coherence. Importantly, even dividing cells show just a slight decrease in instantaneous speed, suggesting they are being carried by their neighbors when they are busy dividing, thus exhibiting collective migration. Similarly, creating mosaic expression patterns by injecting plasmid DNA and performing transplants, it was shown that leading edge cells and posterior cells utilize similar molecular mechanisms and signaling pathways to migrate. Specifically plate cells depend on the same PI3K-pathway to migrate properly.

Previous work conducted on prechordal plate migration has suggested that cells should be able to migrate individually if they all have the same migrating behaviors and use the same signaling pathways (Kai et al., 2008; Tada and Kai, 2012), however single cells are not able to

migrate, presumably because they have lost their plate identity. In order to further address this idea, Dumortier and colleagues aimed to study single cell migration of plate cells, which retain prechordal plate identity. Using *gsc* as a marker for precordial plate cells, it was determined that single cells with deactivated *casanova* (needed for complete precordial plate migration) transplanted into a host embryo would not migrate towards the animal pole. In fact, single cells stay around their position or migrate backwards until joined by the front plate cells, at which time they can migrate towards the animal pole. This suggests single cells are able to migrate independently, but do not actually migrate to their correct position unless in a collective cohort of cells, supporting the idea of collective migration. Furthermore, by analyzing the actin dynamics of these isolated cells, it was determined that prechordal plate cells have intrinsic polarity and protrusive activity that is dependent upon cell-cell interaction for orientation, thus dependent upon CIL. Transplanted cells reorient actin protrusions towards the animal pole when they come into contact with endogenous plate cells.

To determine the molecule responsible for cell-cell contact mediated polarization, E-cadherin became a candidate because it is expressed in migrating prechordal plate cells. E-cadherin has been shown to be important for protrusion formation during migration. E-cadherin morphant cells transplanted into WT hosts showed fewer membrane protrusions and those cells that did have protrusions were not oriented towards to animal pole like those of control injected embryos, suggesting E-cadherin is required for polarized cell protrusions towards the animal pole. Additionally, using dominant negative forms of Dsh, RhoA, Cdc42 and Rac1, it was shown that Wnt/PCP and RhoA/Rac1 pathways play an important role in orienting cells and cell protrusions, together with E-cadherin, during contact inhibition of locomotion in collective migration (Dumortier et al., 2012a).

Conducting all the above experiments allowed Dumortier and colleagues to propose a model for collective migration in precordial plate cells. Cells migrate autonomously using actin-

rich extensions, which are mediated collectively by E-cadherin cell-cell contacts. E-cadherin cell contacts promote intrinsic polarity in a migrating group through Dsh/PCP- and Rac1-dependent processes and also CIL behavior from E-cadherin contacts allow directionality of collective groups of cells. In all, E-cadherin mediated contacts allow groups of cells to migrate in a directed manner and collectively through CIL.

Role of CIL in Neural Crest migration

Migration of neural crest cells relies on cell-cell contacts to regulate directional movement of cells. This directional movement is influenced by CIL. Moreover, N-cadherin dependent cell-cell contacts are required for the molecular component of CIL as shown in *Xenopus* embryos (Carmona-Fontaine et al., 2008).

Specifically, Xenopus neural crest cells are used as a model to study the molecular mechanism regulating CIL (Theveneau et al., 2010). They first use morpholino knockdown and then rescue co-injection experiments *in vivo* to show that an Sdf1-Cxcr4 gradient is required for directional migration of neural crest cells (NCCs). Then they went on to further confirm that Sdf1 is a specific NCC chemoattractant using *in vitro* bead assays soaked in either Sdf1, PBS, dominant-negative (DN)-Cxcr4 or *cxcr4* knockdown (Theveneau et al., 2010). In addition, they visualized whether cells migrated together in cluster or individuals in close juxtaposition to each other using fluorescent labeling and confocal microscopy. They determined that cells were migrating as a group and only outer cells showed large protrusions and cells within the group did not form protrusions with each other. Additionally, outer cells displayed distinct polarity with centrosomes in an off-center orientation and microtubules growing towards the free edge of the group of cells (thus not towards the other cells in the group). Next they studied if response to Sdf1 chemo attraction was dependent upon cells being in a group. When cells are dissociated and re-associated with each other, they migrate towards Sdf1 just as efficiently

as cells that were not dissociated, showing that breaking cells apart has no effect on ability to respond to Sdf1, as long as they are able to reorient back into a group. More importantly, individual cells, dissociated and plated singly, do not respond to Sdf1 chemoattraction, supporting the idea that collective cell interactions are critical for chemotaxis, and thus important for directed migration. In summary, they showed that NC cells require chemotaxis to Sdf1 for directed migration and that cells must be part of a group for the chemotaxis response. The authors coined this as “collective chemotaxis”.

Theveneau and colleagues wanted to further characterize NCC group’s ability to respond to chemotaxis in order to sustain directive migration. In order to achieve this, they examined the chemotactic response of cells in three different conditions: 1) low density of individual cells with no contact, 2) individual cells having only transient cell-cell contacts and 3) high density of individual cells and cell clusters interacting with each other. They found as cell density increases, response to chemotaxis also increased, and that individual cells with transient contacts are able to respond to Sdf1 unlike isolated cells with no cell interactions. These results suggest that NC cells can always sense Sdf1, but are only able to respond to Sdf1 if they have interactions with each other. Moreover, even though the response is more efficient with larger groups of cells, small groups or clusters can still respond, suggesting that cell-cell interactions are essential for a chemotactic response and not the specific size of the group present. Furthermore, using *in vitro* and *in vivo* chemotaxis assays, they were able to show that wild-type NCCs display collective cell migration, allowing them to rescue mutant cells thus forming a cluster and the whole group moved in a net forward motion.

Next, Theveneau and colleagues aimed to determine the particular roles of chemotaxis and cell-cell contact during NCC directed migration. They used confocal imaging of single cells verses groups of cells, showing that single cells exposed to Sdf1 make numerous small unstable protrusions randomly. In contrast, cells in a group have a fewer, but larger and more

stable protrusions, independent of Sdf1 presence. This suggests that chemoattraction does not promote protrusion formation per se, but may have a role in indirectly stabilizing cell protrusions. Cell-cell contact reduces protrusion formations between cells, thereby promoting large stable protrusions to form at the front of cells or on the edges of clusters.

To specifically investigate how stable protrusions are formed in NC migration, FRET analysis was utilized. Past studies have shown that small Rho GTPases, Rac1 and Cdc42 play a role in protrusion formation (Ridley, 2003) and while Rac1 activity has been shown to be required specifically in NCC migration, they suggest there is no evidence of Cdc42 involvement in NCC migration. FRET analysis shows that control cells with normal Sdf1 conditions show polarized Rac1 activity, having high concentrations around the free edge of clusters of cells and low concentrations at sites of cell-cell contacts. Additionally, single cells show no cell polarization and 75% show no Rac1 polarity, suggesting that Rac1 polarization is dependent upon cell-cell contacts within a group and not the presence or absence of Sdf1. Sdf1, however, does amplify the polarity of cells at the free (migrating) edge of cell clusters but has no effect on inner cells of a cluster.

After determining that directed migration is attributed to cell-cell contacts, it was necessary to determine the cell adhesion molecule contributing to cell-cell contact. Using in situ hybridization and immunostaining in *Xenopus* embryos, it was shown that N-cadherin is present in pre-migratory and migratory NC cells, making it a candidate molecule for mediating cell-cell interactions during directed NC migration. Using antisense N-cadherin morpholino injections and also over-expression of full length N-cadherin shows dramatic defects in cell migration, suggesting that N-cadherin levels must be tightly regulated for normal NC migration. Furthermore, by transplanting labeled rhodamine-dextran NC cells into unlabeled host embryos, they were able to confirm that N-cadherin localizes to cell-cell junctions in migrating NCCs. Because N-cadherin is localized to cell-cell contacts in migrating NC cells and changing

levels of N-cadherin prevents normal migration, N-cadherin appears to be a critical component to functional cell contacts during NC cell migration. Moreover, in the presence of N-cadherin-blocking antibody, NCCs lose their attraction toward Sdf1, similar to cells spreading randomly without the presence of Sdf1. This lead to the hypothesis that N-cadherin is required specifically at sites of cell contact for contact inhibition of protrusions, allowing response to chemotaxis.

In order to test this hypothesis, they observed the protrusive activity of N-cadherin morpholino injected NC cells. These cells were highly motile and made more protrusions than NC cells in control injected embryos. Also, morphant cells made protrusions on top of each other and showed extensive overlapping, suggesting that N-cadherin plays a critical role in inhibiting protrusions between neighboring cells. Thus without N-cadherin, NC cells are blind to their neighbors. Examination of Rac1 activity in N-cadherin blocking antibody treated NC cells, Rac1 activity was decreased in outer cells and Rac1 activity was increased at regions of cell contact. This suggested that N-cadherin is critical for maintaining cell polarity by inhibiting Rac1 activity at cell-cell contacts, which in turn increases Rac1 activity at the free edge of the cell.

Because knocking down N-cadherin leads to extra, random protrusions, it was necessary to specifically test N-cadherin's role in CIL using single cell collisions assays and explants invasion assays. Control NC cells showed an intense change in direction after collisions occurred and no ability to invade each other, whereas N-cadherin inhibited cells showed no change in direction after collision and extensive invasion and overlapping with each other. These results demonstrate that the presence of functional N-cadherin is required for NC cells to display CIL.

The extensive experiments performed by Theveneau and colleagues suggest that migrating NC cells required cell-cell contact for persistent directed migration. These cell-cell

contacts are also required for chemotaxis of NC cells, specifically for chemoattraction to Sdf1. N-cadherin is a critical cell adhesion molecule involved in migrating NC cell-cell contacts which promotes CIL behavior through the regulation of Rac1 activity (Theveneau et al., 2010).

CIL in Wound Healing

Although the specific role of cadherin in CIL of wound healing has not been exclusively studied, it has been proposed that Cadherins are involved in CIL during wound healing. Specifically, the role of E-cadherin has been studied in epithelial embryonic wound healing.

Experiments conducted by Hunter and colleagues suggest that endocytic machinery is immediately recruited to the site of wound margins and is necessary to reduce the amount of E-cadherin present at wound edges. Additionally, calcium signaling in epidermal cells promotes polarized recruitment of endocytic molecules, thus allowing wound repair to occur. Although this is not a direct implication of cadherin molecules with CIL, one could propose that E-cadherin reduction is a mechanism by which CIL occurs in wound healing, thus when E-cadherin is impaired, epithelial wound closure is also impaired (Hunter et al., 2015).

CIL in Cancer Metastasis

Although the pathology and mechanisms of cancer cell migration do not directly correlate with FBMN migration, it is important to highlight the role of CIL in cancer cell dynamics as a model for CIL studies. CIL has been discovered to be lost in some cancer cells, a phenomena established long ago with initial CIL studies conducted by Abercrombie and Heaysman nearly 50 years ago When migrating cancer cells contact each other, they do not stop their migration but continue to proliferate and grow on top of one another, forming masses of cells (tumors) (Abercrombie, 1979). More recent studies using prostate cancer lines co-cultured with fibroblasts suggested that combinations of Eph receptors and reciprocal ephrin

ligands between surrounding cells influences whether cancer cells will be restricted by CIL or not (Astin et al., 2010). They suggest that specifically, CIL in some cancer cells is mediated by EphA signaling.

FACIAL BRANCHIOMOTOR NEURONS (FBMNs)

Anatomy and Development of Zebrafish Hindbrain Motor Neurons

To investigate the cellular and molecular mechanisms regulating neuron migrations, we use the tangential migration of a population of neurons located in the vertebrate hindbrain, facial branchiomotor neurons (FBMNs) as a model system.

During early development, the neural tube becomes segmented into three distinct regions along the anterior-posterior axis: forebrain, midbrain, and hindbrain (Fig.1A). The most posterior or caudal section, the hindbrain, also referred to as the rhombencephalon, is further partitioned into eight compartments called rhombomeres (Chandrasekhar, 2004). Each rhombomere expresses a distinct set of *Hox* genes, each giving rise to a unique set of motor neurons. For instance, motor neurons innervating different muscles in the head, neck and gut are born in distinct rhombomeres depending on their function(Chandrasekhar, 2004). These cranial motor neurons make up the motor component of several cranial nerves that exit the midbrain and hindbrain. These cranial motor neurons are classified as somatomotor, branchiomotor, or visceromotor neurons depending on their target of innervation. Somatomotor neurons control eye and tongue movement, visceromotor neurons form part of the parasympathetic nervous system and innervate post-ganglionic parasympathetic neurons, and branchiomotor neurons (BMNs) are a type of cranial motor neuron controlling jaw movements, facial expressions, the larynx and the pharynx. Examples of branchiomotor neurons include the trigeminal motor neurons, born in rhombomere 2 (r2) and r3, that make up the motor

component of trigeminal nerve (cranial nerve V) and innervate muscles that move the jaw for chewing. In addition, facial branchiomotor neurons (FBMNs), born in rhombomere 4, make up the motor component of the facial nerve (cranial nerve VII) and innervate muscles responsible for facial expressions in humans (Fig. 1A-B).

BMNs, as opposed to other cranial motor neurons, are an ideal model system to study for several reasons. BMN development is evolutionarily conserved between zebrafish, mouse and chick. They can be easily labeled with retrograde fluorescent tracers, giving insight to their development and organization. There are a wide variety of genetic, genomic, molecular and embryological tools available to study BMNs in the above model organisms. Thus, these neurons represent an easily accessible model neuron population to investigate the molecular cues guiding development, migration, path finding and innervation. Specifically, we use FBMNs as a model for tangential migrations in the central nervous system. We do this in zebrafish to take advantage of transgenic tools that permit the visualization of cranial branchiomotor neurons, genetic tools to express genes of interest in BMN populations, and the transparency of zebrafish embryos for live imaging.

Visualizing FBMNs Using the islet-1 Promoter

In order to study FBMNs, we utilize transgenic zebrafish expressing green fluorescent protein (GFP) under control of the *islet-1* (isl1) promoter: *Tg(Isl1:GFP)*. *Islet-1* is a member of the LIM/homeobox gene family and it is expressed in all post-mitotic motor neurons and sensory neurons early in their development. Previous studies identified the minimal enhancer region within the *isl1* promoter responsible for driving expression specifically in cranial motor neurons. This enhancer element, termed zCREST1, was then used to drive expression of GFP and a stable transgenic line was created (Higashijima et al., 2000). This stable transgenic line labels several cranial motor neuron populations within the developing zebrafish hindbrain. This

transgene marks trigeminal BMNs (TBMNs), which arise in r2 and r3, FBMNs that are born in r4, and large columns of vagus motor neurons born in r8 (Chandrasekhar, 2004) (Fig. 1B). All of these cranial motor neuron populations undergo migration during development. TBMNs are born in the medial neural tube or r2 and r3 and migrate laterally to their final destination, FBMNs migrate from r4 to r6 in mammals and r6/r7 in zebrafish, and vagus motor neurons undergo a dorsolateral migration to their final destination in large dorsal columns (Chandrasekhar, 2004).

The *Tg(isl1:GFP)* line also expresses GFP in cells that are not motor neurons. It is important to highlight how these cells are distinguished from FBMNs when studying FBMN migration. Lateral line efferents (LLe) innervate hair cells in the lateral line and otic efferents (OLe) innervate hair cells in the inner ear. Both of these cell populations are both born in r4 and migrate into r6 and r7, similar to FBMNs (Fig. 1A). Their axons also exit at r4, like FBMNs, but quickly diverge from the facial nerve to path find to the lateral line and otic vesicle, respectively. Currently, there are no molecular markers that help distinguish FBMNs from Ole neurons. The only way to differentiate between FBMNs and Ole neurons is using morphological criteria. Unlike FBMNs, whose dendrites project laterally, Ole neurons have a large dendrite that grows to the contralateral hindbrain crossing the floor plate. The presence of these large processes allows us to differentiate them from FBMNs. In addition, there is a second population of lateral line efferents, called posterior lateral line efferents (PLLe) that are born in r6, instead of r4. The PLLe neurons migrate caudally from r6 into r7 and are GFP-positive in *Tg(isl1:GFP)* embryos. These PLLe neurons could easily be mistaken for r4-derived FBMNs or r4-derived Ole neurons; however, their axons project out of r6, instead of r4, and innervate neuromast hair cells in the posterior lateral line (Chandrasekhar, 2004; Sapède et al., 2005). Taken together, knowledge of the development and neuroanatomy of cranial motor neurons highlighted in the *Tg(isl1:GFP)* zebrafish allow us to effectively image and quantify

FBMN migration *in vivo*.

For the purposes of this thesis, r4-derived FBMNs and Ole neurons will be referred to simply as FBMNs for simplicity, unless a more detailed description is necessary. Due to the different origin of r6-derived PLLe neurons, these neurons will be continuously highlighted to not be confused with r4-derived FBMNs.

FBMNs: Brief Anatomy and Migration

Facial Branchiomotor Neurons (FBMNs) are a distinct population of motor neurons born in the ventral hindbrain and form the motor component of the facial nerve, innervating second pharyngeal arch muscle derivatives. In humans, the facial nerve innervates muscles of smiling and frowning. In zebrafish, the facial nerve innervates muscles that insert on the jaw and muscles that move the opercle, the largest bone of the operculum that covers the gills. Damage or mutations resulting in defects to the facial nerve are associated with syndromes such as Bell's Palsy and Mobius syndrome (Wanner et al., 2013). FBMNs undergo tangential migration from their birthplace to their final destination. These neurons, as well as their characteristic tangential migration, are conserved from fish to mammals (Chandrasekhar, 2004). In zebrafish, FBMNs are born in r4 at 16 hpf in ventral portion of the neural tube, in the motor neuron progenitor domain, close to the floorplate. By 18 hpf, the earliest born neurons begin to migrate caudally along the ventral aspect of the neural tube, in and amongst the neuroepithelal cells. As they migrate caudally or posteriorly, they leave their axons behind them, where they will exit the hindbrain in r4. These axons are actively path finding as the cell soma is actively migrating posteriorly, indicating that these cells are highly motile in many different places and may also suggest that the molecular cues that regulate both cell soma movement and axon path finding are likely to be different. It has been suggested that the first axons laid and other pre-existing axon tracts are required for proper tangential migration.

Wanner and Prince reported that like other cell types, FBMN populations have a pioneer neuron, which is the first FBMN to migrate out of r4 leaving behind its corresponding pioneer axon. They suggested this axon is required for proper early migration of follower neurons out of r4. Additionally, they noticed FBMNs also migrate in close proximity to axons of the medial longitudinal fasciculus (MLF), which is one of the first axon tracts to form in zebrafish and extends throughout the ventral nervous system. Wanner and Prince also suggested once FBMNs have migrated out of r4, they use the MLF to migrate through r5 and into r6details discussed further below) (Wanner and Prince, 2013b). Earliest born FBMNs sometimes migrate into r7, while the rest migrate into r6 (Fetcho et al., 2008; Grant and Moens, 2010). Once neurons have migrated to r6/r7 they migrate laterally within those rhombomeres (Chandrasekhar, 2004; Song, 2007) and finally extend dendrites laterally (except of course the Ole contralateral dendrite). FBMNs continue to arise in r4 and migrate into r6 until migration is complete around 48 hours post fertilization.

Molecular mechanisms of FBMN Migration

The migration of FBMNs is a highly regulated process by several molecules, both intrinsic and extrinsic, including but possibly not limited to transcription factors, members of the PCP pathway, cell adhesion molecules (CAMs) and chemotactic ligands/receptors. Migration is governed by an interplay of all of the above factors, making FBMN migration quite complex. As one can imagine, defects in any of these above cues results in a range of migration defects and it is important to note the specific role of each.

Transcription factors involved in FBMN migration

The ability of FBMNs to migrate is partially a result of their location within the developing hindbrain. Several studies in vertebrate development have established the hindbrain is

transiently segmented into genetically distinct regions called rhombomeres, which play a critical role in the organization and structure of the vertebrate brain (Guthrie, 1996; Lumsden and Krumlauf, 1996; Prince et al., 1998).Differential gene expression allows each rhombomere to give rise to specific neuronal types, largely controlled by transcriptional regulation (Wanner et al., 2013). Specifically, *Hox* genes are differentially expressed along the anteroposterior plane of the developing hindbrain (Prince et al., 1998). In the case of zebrafish FBMNs, the transcription factor *hoxb1a* is specifically expressed in r4, where newly born FBMNs begin their migration, and is essential for FBMN migration. Knockdown of *hoxb1a* with morpholinos results in a failure in the characteristic posterior migration. Similar results are seen in mouse knockouts of *Hoxb1* (mouse ortholog) (McClintock et al., 2002). At least one downstream effector of Hoxb1 has been identified. A differential expression screen between wild-type and *hoxb1a* morphant hindbrains identified *prickle1b* (*pk1b*), a member of the planar cell polarity signaling pathway, as a gene that is positively regulated by Hoxb1a (Rohrschneider et al., 2007). This suggests that Hoxb1 may regulate neuron migration of FBMNs in part by controlling the expression of planar cell polarity genes.

There are also other transcriptional networks proposed to influence FBMN migration. T-box genes have been linked to many processes during early development, including gastrulation, heart development, and uterine implantation during pregnancy (Naiche et al., 2005). In the hindbrain, *tbx20* is expressed specifically in cranial motor neurons, including FBMNS, but not in the surrounding neuroepithelium in both mice and zebrafish models (Pocock et al., 2008; Song et al., 2006). While hindbrain patterning is normal in *Tbx20* null mouse mutants, neuron migration is disrupted in all branchiomotor neurons. Specifically, FBMNs do not migrate caudally as they do in control mice, suggesting Tbx20 is required for the normal tangential migration of FBMNs (Song et al., 2006). The downstream effectors of Tbx20 are currently unknown. Other transcription factors that have been shown to play a role

in FBMN migration include Gata3, Phox2b, and Ebf-1(Garel et al., 2000; Pata et al., 1999; Samad et al., 2004).

Planar Cell Polarity Proteins Implicated in FBMN Migration

What is PCP?

Planar cell polarity (PCP) is described as a cell contact-dependent method for establishing and maintaining polarity within the plane of a tissue or cell, such as that of an epithelium. Originally, PCP was best described and studied in the formation of *Drosophila* wing hairs, called trichomes, whose development correlates with distinct localization of core PCP proteins. Trichomes are actin-based hairs that form on the distal side of wing epithelial cells and point distally. As development progresses, asymmetric localization of PCP proteins to either the distal or the proximal end of the wing cell allows the trichome to arise in the distal portion. Loss of any of the "core" PCP proteins (discussed further later) cause morphological defects in *Drosophila* wing development resulting in the loss of asymmetric localization of trichomes (Das et al., 2004). Instead, mutation in any of the core PCP genes causes the trichome to form centrally and point in random directions. It is now recognized that PCP proteins are evolutionarily conserved and PCP proteins have been implicated in polarization of many other vertebrate tissues. For instance, PCP is essential for proper alignment of inner ear hair cells, and asymmetric localization of motile cilia in the kupffer's vesicle (node) and floorplate (Wallingford, 2012). PCP has also been shown to be required for motile cells, including convergent extension movements of mesodermal cells and neuron migration.

Aside from polarizing epithelial cells, the PCP pathway is also involved in polarizing groups of cells, such as directing convergence and extension (CE) cell movements during gastrulation. Convergence extension is a critical process during embryogenesis when cells intercalate with each other, become narrow and therefore elongate the body axis (Wallingford,

2006). PCP loss of function mutants in zebrafish and *Xenopus* cause reduced cell intercalation resulting in a shortening of the body axis, a phenotype stereotypic to CE loss of function mutants (Heisenberg and Tada, 2002). Additionally, PCP proteins are also required for coordinated cell movements allowing neural tube closure. For example, experiments in the mouse mutant Looptail, which presents with a neural tube closure defect indicate that this defect results from a mutation in Vangl2. Mutations in other PCP pathway components, such as Dsh and Fzd have also been associated with neural tube defects (Wallingford, 2006).
In order to review the impact PCP proteins have on FBMN migration, it is first important to distinguish the key players. PCP proteins make up two distinct protein complexes and localize separately to cell membranes, which varies depending upon the cellular context. Core PCP proteins include three transmembrane proteins, Frizzled (Fzd), Van Gogh (Vangl), and the atypical cadherin Celsr, and several cytoplasmic adapter proteins, Disheveled (Dsh) Prickle (Pk), and Scribble. The above proteins are regarded as “core” PCP proteins in this cellular context and loss of function mutants in any of these core proteins results in a loss of polarity (Carvajal-Gonzalez and Mlodzik, 2014).

Brief history of PCP pathway proteins in FBMNS

It is now established that PCP components play a role in regulating the migration of neurons such as FBMNs. Several experiments using zebrafish loss of function mutants for Vang-like 2 (Vangl2), Prickle (Pk1a and Pk1b), Frizzled (Fzd3a), Scribble1 (Scrib1), and Celsr (Celsr1/2) have all shown defects in FBMN migration. Bingham and colleagues used an antibody screen of islet-1 stained mutants and discovered that Vangl2 is not only required for CE during gastrulation, but also for development of cranial nVII and nIX neurons (this included FBMNs). Specifically, FBMNs in *vangl2* mutants fail to migrate from r4 to r6/r7, but axon path finding appears to occur normally (Bingham et al., 2002; Jessen et al., 2002). Experiments by

Carreira-Barbosa and colleagues built upon evidence that PCP is critical to FBMN migration. They were able to isolate a zebrafish homologue of *Drosophila prickle (pk)* and knock it down using a morpholino approach. They established that Pk1 is required for FBMN migration and also strongly interacts with Vangl2 to mediate caudal migration (Carreira-Barbosa et al., 2003). Building upon those, experiments with transgenic zebrafish expressing GFP in cranial motor neurons found another novel migration mutant caused by the gene scribble1 (scrib1), a homologue of the *Drosophila* cell polarity gene *scribble.* They also showed that like Pk1, Scrib1 also interacts with Vang2 at the membrane to regulate both CE movements and neuron migration (Wada, 2005). Subsequent experiments by the same group established novel roles for the genes Frizzled3a (fzd3a) and Celsr2 (celsr2) in addition to those previously established by other experiments (such as development of the anterior commissure, and the cortico-subcortical, thalamocortical and corticospinal tracts). Using *fzd3a* and *celsr2* morphants, it was suggested that these genes play an important role in neuroepithelial cells by keeping migrating FBMNs ventrally while migrating through the neural tube. In their absence, FBMNs randomly migrate dorsally and do not reach r6/r7 (Wada et al., 2006a). All of the above studies had established PCP proteins as a critical gene network required for FBMN migration both in FBMNs and the environment; that is, PCP localization and signaling occurs between migrating neurons, between migrating neurons and neuroepithelial cells and also between neighboring neuroepithelial cells themselves. Later experiments using micro-array to establish novel genes specifically within FBMNs discovered the PCP protein Pk1b is expressed specifically in FBMNs and not in neuroepithelial cells (Rohrschneider et al., 2007). The finding that pk1b functions cell-autonomously in FBMN migration was unique in FBMN migration and became an important tool to further study the tangential migration of FBMNs. In addition, several studies have also shown similar results in mouse mutants for the same PCP components (Davey and Moens, 2017; Glasco et al., 2012).

PCP proteins function both cell-autonomously and non-cell autonomously to achieve tangential migration

It is still not completely clear how PCP is influencing cell polarization and migration on a molecular level in FBMNs. Using cell transplantation experiments, it has been established that PCP is required non-cell autonomously and cell autonomously; that is, it is required both in the neuroepithelial cells and *within* the neurons during FBMN migration. At time points of FBMN migration, the mRNA expression of core PCP genes is ubiquitous. Experiments using cell transplantation demonstrated that wild-type neurons are unable to migrate in a *vangl2*, *fzd3a* and *scrib* mutant host embryos, suggesting that PCP is required within environment to regulate caudal migration(Davey et al., 2016). These findings indicate that a planar polarized environment, likely neuroepithelial cells is necessary to promote caudal movement of FBMNs. Further experiments additionally established a cell-autonomous role for PCP proteins in neuron migration. When the reciprocal transplant was conducted, PCP mutant neurons transplanted into host wild-type embryos, ~50% of FBMNs are blocked in r4 and ~50% migrate normally(Davey et al., 2016; Mapp et al., 2010; Walsh et al., 2011). The ~50% of FBMNs that remained blocked in a wild-type environment are consistent with a cell autonomous role for PCP function. However, the ~50% of PCP-deficient neurons that migrate caudally in a wild-type environment suggest that there must be something either in the neuroepithelial environment or within migrating neurons, which allow some PCP-deficient neurons to be rescued.

In order to determine whether neighboring wild-type neurons were responsible for the rescue of PCP-deficient FBMNs, a transplant strategy was devised to place PCP-deficient donor cells into a host whose environment was normal and polarized, yet whose neurons would not migrate caudally. To accomplish this, Walsh et al., made use of *pk1b* mutants.

Because the PCP component Prickle1b (Pk1b) is only expressed within the FBMNs and not the neuroepithelial environment (unlike all other PCP components), *pk1b* mutants display normally polarized neuroepithelial cells but a complete failure in caudal migration of FBMNs (Mapp et al., 2010).Indeed, wild-type cells transplanted into a *pk1b* mutant were capable of caudal migration supporting the notion that the wild-type environment is normal in *pk1b* mutants (Walsh et al., 2011). Interestingly, when PCP mutant host cells (*scrib-/-, nhsl1b-/-,* and *vangl2-/-*) were transplanted into *pk1b* mutant hosts, they failed to migrate out of r4 (Walsh et al., 2011). This observation suggested that the cell type responsible for the rescue of PCP-deficient neurons were neighboring host wild-type FBM neurons. In the absence migrating host neurons, PCP-deficient neurons all remain in r4, despite a normal polarized neuroepithelial environment. These experiments indicated that PCP proteins also function *within* (cell autonomously) FBMNs, and clarify that the reason PCP-deficient neurons sometimes migrate is because they are rescued by neighboring FBMNs themselves. Moreover, the fact that a wild-type neuron can rescue the migration of another neuron (in this case the PCP-deficient neuron), classifies this migration as a collective migration, since one neuron can influence the migration of an adjacent neuron. Taken together, these experiments supports several main conclusions: 1) PCP proteins function both in the neuroepithelial environment and within FBMNs to promote caudal migration, 2) there is a collective mode of migration occurring in FBMNs (that does not require PCP signaling in the rescued cell) to promote sustained, directional migration from r4 to r6/r7 (Walsh et al., 2011).

The Role of Cell Adhesion Molecules (CAMs) in FBMN Migration

Cell migration in general requires regulation of cell adhesion complexes in both migrating cells and the surrounding environment. In order to migrate properly, cells must be able to regulate cell adhesion with similar cell types (homotypic) other cell types (heterotypic),

as well as with the extracellular matrix (ECM). Different experiments conducted in zebrafish have established a role for different families of cell adhesion molecules (CAMs) such as immunoglobulin CAMs (IgCAMs), and Cadherins in neuronal migration.

Cadherins are a family of calcium-dependent CAMs that participate in homophillic binding to each other between neighboring cells. There are several different subfamilies of Cadherin found in vertebrates, including classical type I, classical type II, desmosomal cadherins, seven-pass transmembrane cadherins, protocadherins and large cadherins of the fat and dachsous group (Stemmler, 2008). Cadherins are described as a family of glycoproteins with an extracellular portion, a single transmembrane domain and cytoplasmic domain, with the exception of seven-pass transmembrane cadherins (Derycke et al., 2004). The extracellular portion consists of several cadherin repeats (referred to as EC) and is critical for binding of Ca^{2+} ions to allow intercellular binding and signaling (Derycke et al., 2004; Stemmler, 2008). Without the ability of calcium binding, cadherins cannot function properly and are therefore referred to as calcium-dependent molecules. For the scope of this thesis and the work it represents, focus will be on classic type I cadherins.

Classic type I cadherins include E-cadherin, N-cadherin, P-cadherin and R-cadherin, named for the tissue in which they were first discovered (epithelial, neuronal, placental and retinal, respectively) (Stemmler, 2008). Type I classic cadherins are distinguished by the presence of a His-Ala-Val (HAV) amino acid sequence in the first cadherin repeat on the N-terminus. This HAV motif is absent in other cadherin subfamilies and is required for interaction both homotypically and heterotypically (Derycke et al., 2004; Takeichi, 1995). Additionally, Ca^{2+} functions to link the EC domains together and allow proper morphology of the extracellular portion. Taken together, calcium binding provides structure for the cadherin molecule, whereas the HAV domain on the end of the extracellular portion allows site

recognition for binding. Both of these domains in the extracellular portion of cadherins are required for cadherin-cadherin binding.

Not only are cadherins unique in their extracellular architecture, but their cytoplasmic domains also have functional domains that bind to cytoplasmic adaptors, that in some instances mediate signaling, although this cascade is still somewhat unclear. The cadherin cytoplasmic domain binds to β-catenin, often before it has entered the outer plasma membrane, as cadherin and β-catenin are packaged in the endoplasmic reticulum and transported in a vesicle to the membrane together (Niessen et al., 2011). Once cadherin translocates to the plasma membrane, has bound calcium and participated in homo or heterophilic binding, it can then interact with α-catenin. This forms a protein complex essential for cell adhesion (Aberle et al., 1994). Once α-catenin is bound to β-catenin, it is able to mediate a connection to the actin cytoskeleton and eventually influence cell stability and/or motility depending on the cell type. Additionally, the armadillo repeat family member p120-catenin (p120) also associates with this complex (Stemmler, 2008), which appears to act as a modulator for cadherin activity by regulating turnover and is only recruited to the membrane once cadherins are already present (Derycke et al., 2004; Stemmler, 2008). The association of p120 with the cadherin-β-catenin complex is context dependent and can act to regulate cadherin in two ways. First, p120 can act as a scaffold protein to increase adhesion by clustering cadherins to specific sites on the plasma membrane. Cadherin mutants studied in *Xenopus* and cell culture models showed decreased cadherin clustering when the cytoplasmic portion was absent and purification assays in these models resulted in an overwhelming presence of p120 protein (Yap et al., 1998). Secondly, p120 can regulate cadherin adhesion by controlling cadherin turn over at the plasma membrane. Knockout studies in colon cancer cells with p120 gene mutations show a direct impairment of the cadherin adhesion system, which is later rescued when p120 is added back to the system (Ireton et al., 2002).

An important member of the classical type I cadherin family is N-cadherin (N-cad, Cadherin-2, Cdh2), and is of particular interest regarding neuronal migration in the scope of this thesis. As previously stated, N-cadherin was first discovered in neuronal tissue, specifically in the chick neural retina (Volk and Geiger, 1984) and was later studied in other model organisms including yeast, mice and humans. Specifically, Cadherin-2 (Cdh2; N-cadherin; N-cad) has been extensively studied in regards to FBMN migration. Cdh2 was initially of interest because it is expressed widely throughout the developing nervous system. Previous studies have established that Cdh2 cell-cell interaction is required for neuronal tissue building, neuron migration, axon growth, path-finding and fasciculation and synaptogenesis (Suzuki and Takeichi, 2008). Knockout and knockdown studies causing inactivation of Cdh2 result in severe neural tube defects in both mice and zebrafish (Lele et al., 2002; Radice et al., 1997a), demonstrating its critical role in neural tube morphogenesis, but making it difficult to study later events in neuronal development. Cdh2 function has also been studied using dominant negative (DN) cadherin expression approaches, in which a truncated form is overexpressed in addition to the endogenous form. This approach has been shown in migrating neurons of the lateral reticular nucleus and external cuneate nucleus (LRN/ECN neurons)(Taniguchi et al., 2006). In these studies, DN cdh2 expression results in migration defects of LRN/ECN neurons both *in vivo* and *in vitro*. These studies suggest overexpression of DN form of cadherin sequesters β-catenin, therefore preventing endogenous cadherin from binding and forming its stereotypical cadherin-β-catenin complex to ultimately act on the actin cytoskeleton (Taniguchi et al., 2006). Alternatively, DN cadherin could knockout cadherin function by regulating cadherin turnover. Cultured human epithelial cells expressing DN E-cadherin and N-cadherin showed decreased cell adhesion properties (Nieman et al., 1999). Further examination using metabolic labeling of wild type cells versus dominant negative expressing cells showed

increased endogenous cadherin turnover at the plasma membrane (Nieman et al., 1999), suggesting the DN cadherin creates a cadherin knockdown by up-regulating cadherin turn over, which in turn down-regulates endogenous cadherin function (Nieman et al., 1999).

Although it is known to be important, the specific role Cdh2 plays in neuronal migration was still unclear. Regardless of neural tube defects, studies in *cdh2* mutants and morphants attempted to establish Cdh2's role, but with somewhat conflicting findings. Researchers using *cdh2* mutant embryos suggested Cdh2 is required for cohesion between surrounding neuroepithelial cells, which in turn forces FBMNs to migrate ventrally along the neural tube (Stockinger et al., 2011). In contrast, another study using *cdh2*-depleted zebrafish via morpholino knockdown, suggested that Cdh2 is required for FBMNs to attach to pre-existing axon tracts to properly migrate. In this study, it was suggested that FBMNs used Cdh2 to attach to the pioneer neuron's axon to migrate out of r4. When researchers used laser ablation to get rid of this pioneer axon, neurons did not migrate out of r4 33% of the time (but did migrate 67% of the time). Additionally, when the MLF was prevented from entering the hindbrain by surgical transection, FBMN from r5 to r6 was defective 33% of the time (but normal 67% of the time(Wanner and Prince, 2013b). Both of these studies, however, examine Cdh2 function in the context of a mal-formed neural tube, making it difficult to determine if FBMN migration defects are secondary to neural tube defects. Thus, experiments we conducted aimed to inactivate Cadherin-2 function specifically in FBMNs in the context of a wild-type environment (more details in chapter 2). Our findings suggest that Cdh2 is required cell-autonomously for proper caudal movement. Moreover, we show that Cdh2 is the first molecule to be shown to be required for the collective migration of FBMNs (Rebman et al., 2016).

In addition to Cadherins, IgCAMs have been suggested to play a role in FBMN migration, as IgCAMs have been implicated in a several aspects of neural development

including neuron migration, neuron survival, axon guidance and synaptogenesis (Maness and Schachner, 2007). Specifically, the IgCAM Tag-1/Contactin-2 (Cntn2) has previously been shown to play a role in migration of GABAergic interneurons in mice (Denaxa et al., 2001) and also in migration of FBMNs in zebrafish (Sittaramane et al., 2009). Furthermore, because a defect in Cntn2 (Tag-1) alone has little effect on FBMN migration, it has been suggested that Tag-1 (Cntn2) is required to cooperate with Cdh2 in order to control FBMN cohesion during migration (although further experiments on this should be conducted) (Stockinger et al., 2011). Unpublished data from our own experiments using CRISPR mutants for Cntn2 in zebrafish appears to have no defect in the migration of FBMN (unpublished).

Chemotaxis in FBMN Migration

Chemotaxis is the directional movement of organisms or cells towards a chemical stimulus in their environment and is required in a variety of cellular processes; such as cell migration during development, immune responses and wound healing, and movement of tumor cells in invasive cancers (Charest and Firtel, 2006), as well as the function and survival of single cell organisms (Haastert and Devreotes, 2004).

Studies performed with highly motile leukocyte populations and neutrophils and the model organism *Dictyostelium discoideum*, help support our understanding of directed cell movement required for the above processes (Charest and Firtel, 2006).

During chemotaxis, cells aim to integrate external signaling cues with internal cellular reorganization. In Eukaryotic cells, transducing this chemical signal to a physical change in direction or movement occurs when the cell forms a pseudopod at the leading edge and a uropod at the trailing edge. This process involves a two-part process of physical changes to the cell membrane through actin polymerization at the leading edge of the cell and myosin retraction at the trailing edge of the cell (Charest and Firtel, 2006). It is this rapid

assembly/disassembly gives the cell motile ability (Affolter and Weijer, 2005; Ridley, 2003).

Many experiments have established a role for chemotaxis directing collective cell migration. Of particular interest is the Sdf1 chemokine/Cxcr4 receptor system, shown to be important for collective cell migration of neural crest cells. Studies using *Xenopus* found that migrating neural crest cells were strongly attracted to stromal derived factor-1 (Sdf1) (Theveneau et al., 2010). Since previous studies (discussed above) have established that FBMNs engage in collective migration, it is reasonable to assume chemo taxis plays a part in this, as it does in other systems. Similarly to NC cells, Sdf1 chemokine and its receptor Cxcr4 are both expressed in the developing hindbrain. Sdf1 is expressed in a limited strip in r4 (where FBMNs are born) and Cxcr4b is expressed specifically in FBMNs both when they are born and while they are migrating (Cubedo et al., 2009). Additionally, studies in zebrafish *sdf1a* morphants establish Sdf1 is required for the caudal migration of PLL primordium and facial neurons in the hindbrain (Sapède et al., 2005), although the defect caused by morpholino injection is not well characterized. Further experiments must be conducted to establish the role of Sdf1 and cxcr4 in the context of FBMN collective cell migration.

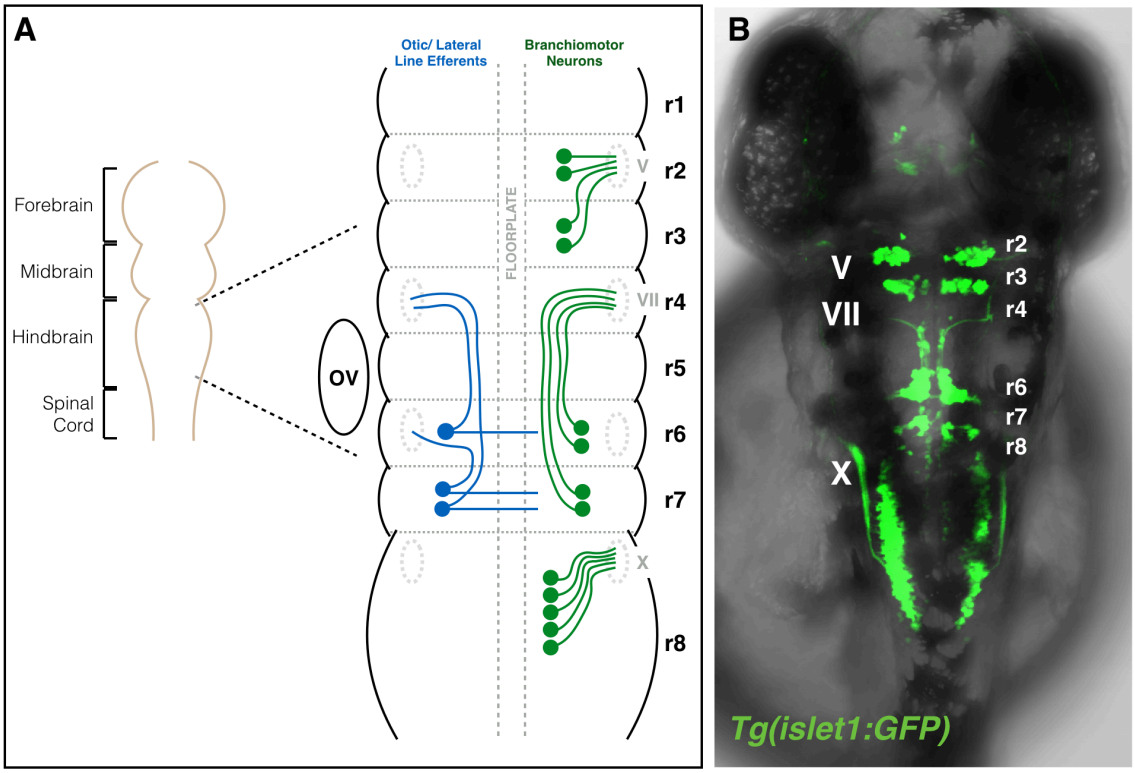

Fig. 1. Organization and anatomy of the hindbrain during development using *Tg(isle1:GFP)* transgenic Zebrafish. (**A**) During vertebrate development, the central nervous system is transiently segmented. One of those segments, the hindbrain (rhomboncephalon), is further segmented into rhombomeres. Branchiomotor neurons are shown in green, which correspond to transgene expressing cells in (**B**). Otic and posterior lateral line efferents are shown in blue, which also may express transgene but can be distinguished from BMNs by presence of a contralateral dendrite. (**B**) Live image of *Tg(isl1:GFP)* transgenic zebrafish showing where GFP is expressed in the hindbrain. V = cranial nerve V, trigeminal nerve; VII = cranial

Chapter 2: Cadherin-2 is Required Cell Autonomously for Collective Migration of Facial Branchiomotor Neurons

Introduction

Neuronal migration is a fundamental step in the assembly of neural circuits that control behavior. Neuron migration integrates multiple cellular and molecular events to coordinate movement from their birthplace to their final destination. In contrast to radial migration in which neurons follow radial glial fibers, tangentially migrating neurons use interactions with other cell types to guide their movement. These interactions may be homotypic, in which a neuron relies on interactions with the same class of neurons, or heterotypic, in which interactions occur between other cell types in their environment to guide their trajectory. Accumulating evidence indicates that some neurons migrate as clusters, chains, or streams, suggesting that they are better described as collective migrations (Marín et al., 2010). Collective cell migration, defined as the coordinated migration of cells, depends on cell-cell interactions between neighbors that contribute to their overall directionality(Mayor and Etienne-Manneville, 2016; Rørth, 2011; Scarpa and Mayor, 2016). The exact nature and importance of cell-cell interactions that lead to collective migration of neurons is unclear, but likely involves the function of cell adhesion molecules.

Cadherins, a family of calcium-dependent cell adhesion molecules, engage in homophilic binding to regulate cell-cell adhesiveness and promote adherens junction formation in stationary epithelial tissues. Cadherin-2 (N-cadherin; Cdh2) is broadly expressed throughout the developing nervous system and Cdh2-based cell-cell interactions are involved in various processes during neural development including tissue architecture, neuron migration, axon elongation, path finding and fasciculation, target recognition and synaptogenesis (Suzuki and Takeichi, 2008). Inactivation of Cdh2 in mice and zebrafish results in severe neural tube

formation defects (Lele et al., 2002; Radice et al., 1997b), making it difficult to determine a role for Cdh2 in later developmental events. However, neuron-specific inactivation of Cdh2 impairs both pia-directed migration of cortical neurons along radial glial fibers by interfering with cell-substrate adhesion (Kawauchi et al., 2010; Shikanai et al., 2011) and leads to impaired tangential migration of neurons from the medial ganglionic eminence to the cortex (Luccardini et al., 2013; Taniguchi et al., 2006) as well as the chain migration of cerebellar granule cells (Rieger et al., 2009), where directional movement is coordinated by Cdh2-based cell-cell contacts.

In this report, we use facial branchiomotor neurons (FBMNs) as a model system to study collective neuron migration. FBMNs are a subset of cranial branchiomotor neurons that are born in the ventral portion of rhomobomere 4 (r4) in the developing hindbrain. These neurons undergo a posterior tangential migration along the ventral portion of the hindbrain to r6 where they form the facial motor nucleus (Chandrasekhar, 2004). During migration, the facial motor axons are laid down behind the migrating neuronal cell bodies that exit the hindbrain at the level of r4 to innervate muscles derived from the second branchial arch (Wanner et al., 2013).

The migration of FBMNs requires heterotypic cellular interactions with the surrounding neuroepithelial cells as well as homotypic interactions with other FBMNs to coordinate their caudal directionality. It is now established that components of the planar cell polarity (PCP) pathway function both non-cell-autonomously (in the environment), as well as cell-autonomously *within* FBMNs to control their caudal trajectory (Bingham et al., 2002; Davey et al., 2016; Jessen et al., 2002; Mapp et al., 2011; Qu et al., 2010; Rohrschneider et al., 2007; Vivancos et al., 2009; Wada et al., 2005; Wada et al., 2006b; Walsh et al., 2011). FBMNs, therefore, display a PCP-dependent mode of migration that requires an interaction with the planar-polarized neuroepithelial cells (Davey et al., 2016; Walsh et al., 2011). FBMNs also

engage in collective cell migration since the migration of one FBMN can influence the migration of another neighboring FBMN (Walsh et al., 2011). Collective migration of FBMNs can be visualized in chimeric embryos generated by cell transplantation in which a wild-type FBMN can promote the caudal migration of a PCP-deficient FBMN (Walsh et al., 2011). Thus, the collective mode of migration promotes the caudal directionality of FBMNs in a PCP-independent manner that requires homotypic FBMN-to-FBMN interactions. The molecular nature of this cell-contact mediated collective neuron migration is not known.

Cadherin-2 is expressed in both migrating FBMNs and the surrounding neuroepithelial cells (Hong and Brewster, 2006; Lele et al., 2002; Stockinger et al., 2011), and Cdh2 depletion has recently been reported to cause defects in FBMN migration (Stockinger et al., 2011; Wanner and Prince, 2013b). Cdh-2 has been shown to promote neuroepithelial cell cohesion that is thought to limit dorsal movement and promote FBMN migration along the ventral aspect of the hindbrain (Stockinger et al., 2011). Wanner and Prince reported that FBMN populations have a pioneer neuron, the first FBMN to migrate, that can direct follower neuron migration in the earliest phase of migration out of r4 and into r5 (Wanner and Prince, 2013b). Indeed, laser ablation of the pioneer neuron or its trailing axon disrupts follower neuron migration (Wanner and Prince, 2013b). Cdh2 knockdown decreases follower FBMN interactions with the trailing pioneer axon, indicating a role for Cdh2 in soma-to-axon interactions that promote caudal movement of follower FBMNs (Wanner and Prince, 2013b). In both studies, a cell autonomous role for Cdh2 function within FBMNs was not directly tested.

Here, we test a role for Cadherin-2 as a cell adhesion molecule mediating the neuron-to-neuron interactions that drive the collective migration of FBMNs. Expression of dominant-negative Cdh2 specifically in FBMNs, and not the surrounding environment, results in a defect in caudal migration. This cell autonomous loss of Cdh2 function leads to random cell movements with resulting impairment in sustained caudal directional migration of FBMNs.

Using mosaic analysis, we demonstrate that the impaired caudal migration of dominant-negative Cdh2-expressing FBMNs is not rescued by the presence of neighboring wild-type FBMNs due to a loss of collective migration. These results are consistent with a model in which Cadherin-2 is required cell-autonomously to drive neuron-to-neuron cell contact-mediated collective migration.

Material and Methods

Zebrafish Husbandry

Zebrafish were maintained following standard procedures and used in accordance with protocols approved by the VCU Institutional Animal Care and Use Committee. All zebrafish used in this study were raised and maintained in our fish facility. Embryos were collected and allowed to develop at 28.5°C to the required stage as described (Kimmel et al., 1995; Westerfield, 2000). Transgenic lines were used as described: *Tg(islet1:GFP)rw0* (Higashijima et al., 2000).

Plasmid DNA Constructs

The *zCrest1* enhancer of the *islet-1* (*isl1*) regulatory elements along with the minimal promoter from the *heat shock protein 70, like* (*hsp70l*) were PCR amplified, blunted and cloned into the EcoRV site in *pTolDest* (gift of Dr. Nathan Lawson) to make *pTol-isl1-hsp70l-DEST*. For simplicity, we will refer to the *isl1-hsp70l* promoter hereafter as the *isl1* promoter. *cdh2ΔEC* was PCR amplified from *pDONR-cdh2ΔEC*, a kind gift of Dr. William Harris (Wong et al., 2012) and fused in frame at the C-terminus with a 5 amino acid linker sequence with PCR amplified *mCherry* with Gibson assembly using primers to add *att* sites at the 5' and 3' ends of the assembled sequence. This sequence was then re-cloned into an entry vector to make *pME-cdh2ΔEC-mCherry.* Using Gateway cloning, this sequence was placed into *pTol-isl1-Dest*

vector to generate *pTol-isl1:cdh2ΔEC-mCherry-Dest*. As a control, we also generated *pTol-isl1:mCherry-Dest.*

Generation of *Tg(isl1:cdh2ΔEC-mCherry)* transgenic lines

Capped mRNA for Tol2 *transposase* was in vitro transcribed using the mMESSAGE mMachine kit (Ambion). DNA encoding each plasmid (50 ng/uL) was co-injected with Tol2 *transposase* mRNA (50 ng/uL) into *Tg(isl1:GFP)* embryos at the 1 cell stage. Founder (F0) embryos were screened for mosaic mCherry expression in cranial motorneurons at 24 and 48 hpf. F0 embryos that were doubly transgenic, displaying both GFP and mCherry expression, were raised to adulthood. Germline transgenic founders were identified by screening F1 progeny for GFP and mCherry fluorescence. Two founders (*vc23*, *vc25*) were isolated, mated with wild-type *Tg(isl1:GFP)* fish, and their GFP- and mCherry-positive progeny were raised to adulthood. To generate embryos containing two copies of the inserted transgene, F1 adults were incrossed and the F2 progeny displaying the brightest mCherry expression were raised to adulthood. To validate that these F2 transgenic fish were homozygous for the *isl1:cdh2ΔEC-mCherry* transgene, each F2 adult was crossed with wild-type *Tg(isl1:GFP)* fish, and the progeny were screened for the predicted expression of Cdh2ΔEC-mCherry in all embryos.

Immunohistochemistry

The following primary antibodies were used. GFP: Mouse anti-GFP (DSHB ;1:100), Rabbit anti-GFP (Torrey Pines; 1:1000), Mouse anti-mCherry (NOVUS; 1:250). Embryos were manually dechorinated and fixed in 4% paraformaldehyde at 4°C overnight, washed in PBST (1× PBS with 0.25% Triton X-100), permeabilized with acetone and incubated for one hour at room temperature in blocking solution (PBST + 4% Goat Serum + 4% BSA). Embryos were then incubated in the corresponding primary antibody and diluted in blocking solution at 4°C overnight. They were then washed in PBST and incubated overnight at 4°C in secondary antibodies. The following secondary antibodies were used at a concentration of 1:200: Alexa

Fluor 488 Goat anti-mouse IgG (H+L) (A11029, Life Technologies), Alexa Fluor 488 Goat anti-Rabbit IgG (H+L) (A11034, Life Technologies). Alexa Fluor 568 Goat anti-mouse IgG (H+L) (A11031, Life Technologies). Alexa Fluor 568 Goat anti-rabbit IgG (H+L) (A11079, Life Technologies). For some embryos, DAPI and Alexa Fluor 488-phalloidin staining was added to the secondary antibody solution. Embryos were washed in PBST and sequentially dehydrated in 25%, 50%, and 75% glycerol in 1× PBS. Yolks were removed using sharpened tungsten wire and embryos were flat mounted on coverslips and surrounded with 75% glycerol medium.

Microscopy and timelapse imaging

Light microscope pictures were obtained on a Zeiss V8 stereomicroscope equipped with an IcC1 camera. Confocal images of immunostained embryos were obtained on an inverted Zeiss Spinning Disk Laser Confocal Observer Z1 using a Zeiss Plan-Apochromat 63X/1.2 W objective, or a Nikon C2 point scanning confocal microscope 40X/1.2 W objective for 48 hpf embryos. For analysis of FBMN motility, embryos were manually dechorionated and mounted in 1.2% low-melting point agarose on the coverslip of a glass bottom dish (Fluorodish; World Precision Instruments). Timelapse imaging was performed at 28.5°C using a heated stage insert. Time-lapse multiple focal plane images were obtained, with each z-stack collected every five minutes for a minimum of one hour. The acquired z-stacks were exported and analyzed using AR-Elements (Nikon) software. Embryo drift was corrected using ND alignment function. For movement analysis of FBMNs, each individual FBMN was manually traced, and the distance between centroids was tracked over multiple frames using AR-Elements software. Cell migration trajectories and instantaneous speed were measured and graphed using Prism Graphpad software.

Heat shock

Fish water was preheated at 37°C. At 6 hpf, embryos were transferred to microcentrifuge tubes containing warmed fish water and placed in the 37°C water bath for 30

minutes. After 30 minutes, embryos were returned to petri dishes containing room temperature fish water and returned to the 28.5°C incubator. Embryos received 2 successive heat shocks spaced 30 minutes apart.

Results

Generation of *Tg(isl1:cdh2ΔEC-mCherry)* transgenic fish

As *cdh2* is expressed in both FBMNs and the surrounding neuroepithelial cells (Lele et al., 2002; Stockinger et al., 2011), we sought to determine whether *cdh2* is required cell-autonomously within FBMNs for their migration. Previous studies, however, have shown that disrupting *cdh2* function in early development results in severe defects in neural tube formation (Hong and Brewster, 2006; Lele et al., 2002). Moreover, chimeric analysis has demonstrated that *cdh2* mutant cells are incapable of contributing to the ventral neural tube due to adhesive differences (Lele et al., 2002). To bypass this, we expressed a dominant-negative form of zebrafish Cdh2, which has a deletion of the cadherin ectodomains 1-4 (Cdh2ΔEC), specifically in FBMNs using the *islet-1* (*isl1*) *zCREST1* enhancer upstream of a heat shock 70-like (*hsp70l*) minimal promoter (hereafter referred to as *isl1* promoter) (Higashijima et al., 2000; Uemura et al., 2005) (Fig 2A). Work in multiple systems has demonstrated that overexpression of this dominant-negative form of Cdh2 results in a non-adhesive phenotype (Jontes et al., 2004; Nieman et al., 1999; Wong et al., 2012).

Using the Tol2 transposition system (Kawakami, 2007), we were able to efficiently generate independent lines of *Tg(isl1:cdh2ΔEC-mCherry*) transgenic fish (Fig. 2B). Two founders (*vc23*, *vc25*) were isolated that produced F1 progeny that showed expression of Cdh2ΔEC-mCherry in cranial branchiomotor neurons (CBMNs) (Fig. 2C-H). These founders were mated with wild-type *Tg(isl1:GFP)* fish, and their GFP-positive, mCherry-positive offspring were raised to adulthood. Adult F1 embryos were raised to sexual maturity and

outbred to wild-type *Tg(isl1:GFP)* fish. Transgenic F2 progeny were generated at approximately 50% (47-53%, $n > 200$ per line), indicating a single insertion site in each founder, and a typical Mendelian inheritance pattern. We observed that the level of transgene expression varied between lines, with line *Tg(isl1:cdh2ΔEC-mCherry)vc23* (referred to as *vc23Tg*) having the lowest and line *Tg(isl1:cdh2ΔEC-mCherry)vc25* (referred to as *vc25Tg*) having the strongest expression. Following an incross of F2 transgenic adults, we also observed that the level of transgene expression was higher in F3 embryos with two copies of the transgene (homozygous) compared to F3 hemizygous transgene carriers (Fig. 3E,H and Fig. 4). Despite expression of dominant-negative Cdh2 in CBMNs, these embryos were viable and fertile.

To confirm that the transgene functioned properly to inactivate Cadherin-2 function, we made use of the minimal promoter from the *heat shock protein 70, like* (*hsp70l*) in our transgenic construct. Under normal physiological conditions, this transgene drives tissue-specific expression in CBMNs due to the function of the *isl1* enhancer but can be used to drive expression in all tissues following heat shock. We reasoned that expression of *cdh2ΔEC-mCherry* in all tissues should phenocopy a *cdh2* mutant. We therefore subjected *vc25Tg* embryos to heat shock at shield stage to coincide transgene activation with the end of gastrulation but before the onset of neurulation. Examination of hindbrain morphology of *vc25Tg* embryos at 24 hours post fertilization (hpf) without heat shock revealed normal hindbrain architecture both in lateral views and in cross-sections (Fig. 2I,K). Following heat shock, *vc25Tg* embryos displayed incomplete fusion of the dorsal neural tube with a mushroom-shaped hindbrain similar to that reported for *cdh2/parachute* mutants (Hong and Brewster, 2006; Lele et al., 2002) (Fig. 2J,L). These results validate the efficacy of our transgene to inactivate Cdh2 function and indicates that overexpression of our transgene does not cause significant off-target phenotypic effects.

We examined transgenic lines for temporal and spatial expression of the *Tg(isl1:cdh2ΔEC-mCherry)* transgene. Both *vc23Tg* and *vc25Tg* transgenic lines displayed expression in CBMNs, consistent with previous reports using the z*CREST1* enhancer of the *islet1* regulatory elements (Uemura et al., 2005) (Figs. 2C-H and Figs 2M-P). Expression of the *cdh2ΔEC-mCherry* transgene was first detectable in trigeminal and facial branchiomotor neurons around 17 hpf, approximately one hour after they are born at 16 hpf. By 24 hpf, in addition to trigeminal neurons and FBMNs, *cdh2ΔEC-mCherry* transgene expression can be detected in most CBMN populations, including oculomotor, trochlear, r4-derived otic and lateral line efferents (Ole), r6-derived posterior lateral line (PLL) efferents, and vagal neurons (Fig. 2O,R). In *Tg(isl1:cdh2ΔEC-mCherry)* fish, expression of Cdh2ΔEC-mCherry is apparent in FBMNs but was not detectable in neuroepithelial cells in the hindbrain (Fig. 2O,R). Image analysis revealed an almost perfect overlap of *Tg(isl1:cdh2ΔEC-mCherry)* expression with *Tg(isl1:GFP)* expression in FBMNs (99.4%, *n* = 800 neurons, 7 embryos). These stable transgenic lines allowed us to examine the cell-autonomous role of Cdh2 in FBMN migration.

Cadherin-2 acts autonomously to control caudal tangential migration of FBMNs

To determine whether Cdh2 is required cell-autonomously for neuron migration, we first examined the caudal migration of FBMNs in *Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos at 24 hpf. In wild-type *Tg(isl1:GFP)* embryos, FBMNs are in the process of migrating caudally from r4 to r6 in wild-type (Fig. 2M,P). In contrast, FBMNs in *Tg(isl1:cdh2ΔEC-mCherry)vc25* are specified correctly in r4 but have largely failed to exit r4 by 24 hpf (Fig. 2N,O,Q-S). We quantified the number of FBMNs migrating in *Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos at 38 hpf, a timepoint in which migration is largely complete. In wild-type *Tg(isl1:GFP)* embryos, FBMNs have migrated properly to r6 (*n* = 678, 6 embryos) (Fig. 3A-C). In embryos hemizygous for either *vc25Tg* or *vc23Tg* transgene, FBMNs exhibit a severe defect in their caudal migration out of r4. In *vc25Tg* hemizygotes, we found that most FBMNs (68.5%, *n* =

589, 6 embryos) fail to exit r4, with a subset that migrates sparingly into r5 (28.2%), and only a small proportion of FBMNs (3.2%) that migrate into r6 (Fig. 3D-F). Embryos hemizygous for the *vc23Tg* transgene also display a defect in caudal migration, albeit less severe than that seen in *vc25Tg* embryos (Fig. 4). The severity of the migration phenotype correlated with the level of transgene expression between the two lines (Figs. 3E and 4). To determine whether transgene dosage would affect phenotype severity, we analyzed embryos carrying two copies (homozygous) of the dominant-negative Cdh2 transgene. In both *vc25Tg* and *vc23Tg* homozygous embryos, the defect in caudal migration was more severe than in embryos hemizygous for the transgene (Figs. 3G-I and 4). In homozygous *vc25Tg* embryos that exhibit the highest level of transgene expression, we found that almost all FBMNs (87.1%, *n* = 7109 neurons, 56 embryos) failed to exit r4 (Fig. 3G-I). The correlation between phenotype severity and transgene expression underscores the importance of expression levels when using a dominant-negative approach.

Although the vast majority of FBMNs remain in r4 in homozygous *vc25Tg* embryos, we consistently observe a small proportion of FBMNs (12%, 855/7109 neurons, 56 embryos) that migrate caudally out of r4 and into r5 but not further. This suggests that FBMNs retain limited capacity for caudal movement in the absence of Cdh2 function.

We infrequently observed a small number of FBMNs that successfully traversed into r6 (0.8%; 57/7109 neurons, 56 embryos). These escaper FBMNs that migrate into r6 are characterized by the presence of a trailing axon that extends back towards r4 distinguishing them from r6-derived PLL efferents (marked by asterisk)(Figs. 3G-I and Fig. 5). We quantified the number and distribution of these escaper neurons per embryo. We found that many homozygous *vc25Tg* embryos (41%, *n* = 56 embryos) had no escaper FBMNs at all (Fig. 5). If present, escaper FBMNs were often only on one side of the embryo as one or two cells (45%, *n* = 56 embryos) (Fig. 5). We only found escapers on both sides of the embryo in a minority of

homozygous *vc25Tg* embryos, either as one neuron on each side (5%, *n* = 56 embryos), or one neuron on one side and two on the other (9%, *n* = 56 embryos). Using image analysis, we verified that escaper FBMNs in r6 were Cdh2ΔEC-mCherry-positive (Fig. 5).

To ensure that this defect was not due to a delay in migration, we quantified the number of FBMNs migrating in wild-type (*n* = 8 embryos) and homozygous embryos *vc25TG* at 48 hpf, a timepoint when FBMN migration is complete. Again, we found that almost all FBMNs (89.8%, 1314/1464 neurons, 11 embryos) failed to exit r4 with no increase in the percentage of escapers that reached r6 at this later timepoint in homozygous *vc25Tg* embryos, suggesting that this defect is not due to delayed migration (Fig. 6). Taken together, our results demonstrate that Cdh2 function is required cell-autonomously within FBMNs for their proper efficient caudal migration.

Cdh2 mediates directionality of FBMN migration

In previous studies, Cdh2 was shown to be required for proper axonal pathfinding of spinal cord motorneurons (Brusés, 2011). We therefore examined axonal pathfinding in *Tg(isl1:cdh2ΔEC-mCherry)vc25* fish. Analysis of confocal images from lateral views of *vc25Tg* embryos showed no gross abnormality in the peripheral pathfinding of FBMN axons, or the axons of other CBMNs compared with wild-type embryos (Fig. 7A,B). This suggests that cell autonomous loss of Cdh2 in FBMNs does not affect axon extension or growth cone steering in the periphery but has a specific effect on the positioning of FBMN cell bodies.

Examination of lateral views of *vc25Tg* embryos showed that Cdh2ΔEC-mCherry-expressing FBMN cell bodies that fail to exit r4 lie in an aberrantly dorsal position (Fig. 7B). This was confirmed in confocal images of hindbrain cross sections at 48 hpf from wild-type and homozygous *vc25Tg* embryos. First, consistent with the observation that our dominant-negative transgene was only expressed in cranial motor neurons, the development of the neural tube and overall organization of the neuroepithelial cells was normal in *vc25Tg* embryos

(Figs. 2K and 7C,D). Secondly, Cdh2ΔEC-mCherry-expressing FBMN cell bodies, that fail to migrate caudally out of r4, are instead found in an aberrantly apical and dorsal position within r4 as compared to the normal ventral positioning of FBMNs within r6 of wild-type embryos (Fig. 7C,D). These results show that cell autonomous loss of Cdh2 function within FBMNs leads to ectopic positioning of FBMNs within r4.

To determine the basis for the neuron position changes seen in *Tg(isl1:cdh2ΔEC-mCherry)vc25* fish, we recorded timelapse movies of FBMN movement in wild-type and homozygous *vc25Tg* embryos. We recorded short 35-minute movies at one frame every 5 minutes between 18 and 22 hpf and tracked the cell movements of individual FBMNs at each of the 7 timeframes. Cell tracings reveal that FBMNs in wild-type embryos exhibit sustained directed migration in the posterior (caudal) direction, whereas FBMNs in homozygous *vc25Tg* embryos migrate randomly with many directional changes (Fig. 7E,F). Consistent with these observations, the total caudal displacement of wild-type FBMNS was significantly larger than the overall caudal displacement of Cdh2ΔEC-mCherry-expressing FBMNs (Fig. 7G,H). Although inactivation of Cdh2 did not inhibit cell motility, we did quantify a small statistically significant decrease in instantaneous speed of FBMNs in *vc25Tg* embryos (Fig. 7I). Together, these findings suggest that Cdh2 is not essential in FBMNs for cell motility, but rather helps to coordinate sustained directed caudal migration of FBMNs.

Cadherin-2 is required for collective FBMN migration

Studies of many cell types that engage in collective migration indicate that collectiveness is borne from cell-cell interactions, and thus coordinated migration stems from interactions with their neighbors (Arboleda-Estudillo et al., 2010; Rørth, 2011; Theveneau and Mayor, 2012b). Our previous work demonstrated that FBMN migration can be characterized as a collective cell migration, in which one FBMN can influence the migration of a neighboring neuron (Walsh et al., 2011). FBMNs migrate caudally because they engage both PCP-dependent and collective

modes of migration. A PCP-deficient FBMN can still be directed caudally because it can respond to cell-cell contact mediated collective migration from a neighboring wild-type FBMNs in chimeric embryos (Davey et al., 2016; Walsh et al., 2011). Thus, we sought to determine whether Cadherin-2 is a candidate cell adhesion molecule controlling cell contact-mediated collective migration of FBMNs.

To address this, we used Tol2-mediated transient transgenesis to generate F0 mosaic embryos in which a subset of FBMNs express *cdh2ΔEC-mCherry* under control of the *isl1* promoter (*Tol2-isl1:cdh2ΔEC-mCherry-pA-Tol2*) adjacent to non-expressing wild-type FBMNs. We reasoned that if Cdh2 function is required for collective migration, then *cdh2ΔEC-mCherry*-expressing FBMNs would not be influenced or 'rescued' in their migration by neighboring wild-type FBMNs. As a control, we expressed *mCherry* mosaically in *Tg(isl1:GFP)* embryos. We found that 90.3 ± 11.1% of mCherry-expressing FBMNs migrated normally into r6 in wild-type *Tg(isl1:GFP)* embryos (*n* = 22 neurons; 6 embryos) (Fig. 8A-C). In contrast, we found that 78.1 ± 17.6% of Cdh2ΔEC-mCherry-expressing FBMNs remained in r4, with 18.6% remaining in r5, and only 3.3% reaching r6 (n = 84 neurons in 8 embryos) (Fig. 8D-F). This defect in caudal migration is not improved compared to that seen in stable *Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos alone, suggesting that the presence of neighboring wild-type FBMNs has no influence on the caudal migration of Cdh2ΔEC-mCherry-expressing FBMNs (Figs. 3G-I and 8D-F). Interestingly, we noted that wild-type FBMNs do not migrate as efficiently in embryos expressing Cdh2ΔEC-mCherry mosaically, as compared to control embryos (Fig. 8F). This suggests that having a subset of FBMNs that cannot engage in collective migration impacts the overall caudal directionality of the group of wild-type FBMNs. Taken together, these results demonstrate that Cdh2 is required for FBMN-to-FBMN cell contact mediated collective migration of FBMNs.

Neuron-specific inactivation of Cadherin-2 leads to aberrant positioning of other neuronal populations

To determine whether expression of dominant-negative Cdh2 affected the migration of other neuronal cell types, we examined the cell soma positioning of other CBMNs in *Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos. In wild-type embryos, trigeminal branchiomotor neurons are born in r2 and r3 and make up the anterior (Va) and posterior (Vp) trigeminal motor nuclei, respectively (Chandrasekhar, 2004). The trigeminal motor neuron cell bodies are located medially early in development and then migrate laterally to their final positions (Chandrasekhar, 2004). At 48 hpf, trigeminal neurons in r2 can be seen undergoing their lateral migration in wild-type embryos but not in *Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos (Fig. 9A,B). Vagus motor neurons are born ventrally and undergo a dorsolateral tangential migration to reside in two dorsal columns known as the dorsolateral motor nucleus (dlX) of the vagus nerve as well as two smaller medial columns called the medial motor nucleus (mmX) of the vagus nerve (Fig. 9D). However, vagus motor neurons in *vc25Tg* embryos fail to coalesce into distinct dorsal and medial motor nuclei, and instead are widely distributed across the mediolateral aspect of the caudal hindbrain (Fig. 9C,D). Interestingly, the pathfinding of trigeminal and vagal axons in the periphery is grossly normal in *Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos compared to wild-type controls, indicating that neuron-specific inactivation of Cdh2 leads to a specific effect on cell body positioning of CBMNs (Fig. 3A,B). We also found that inactivation of Cdh2 lead to a defect in the positioning of hindbrain efferent neurons. PLL efferent neurons that innervate neuromasts in the posterior lateral line are born in r6 and migrate caudally into r7, while their axons exit the hindbrain at r6 (Chandrasekhar, 2004; Higashijima et al., 2000; Sapède et al., 2005). In *Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos, many PLL efferents display a partial migration defect with some PLL efferent cell

bodies remaining in r6 (Figs. 2M-S and 3A-I). Taken together, Cdh2 function is required cell autonomously within multiple cranial motor neuron populations for proper neuron migration.

Discussion

In this report, we define a cell autonomous role for Cadherin-2 in mediating the collective migration of FBMNs. We first generated stable transgenic lines expressing dominant-negative Cdh2 specifically within FBMNS and not the surrounding neuroepithelial cell environment. We show that Cadherin-2 is required cell autonomously in FBMNs for sustained migration in the caudal direction. We found that expression of dominant-negative Cdh2 in FBMNs leads to random, non-directed migration, suggesting that Cdh2 is not required for motility, but promotes a coordinated caudal trajectory of FBMNs. Using mosaic analysis, we demonstrate that dominant-negative Cdh2-expressing FBMNs are unable to be 'rescued' by neighboring wild-type neurons, supporting a model in which the collective cell behaviors of FBMNs are driven by Cdh2-based neuron-to-neuron interactions.

Cadherin-2 controls caudal directionality

During neural development, Cadherin-2 is required for the proper morphogenesis of the neural tube (Hong and Brewster, 2006; Lele et al., 2002; Radice et al., 1997b). The severe neural tube formation defects seen in Cdh2 knockouts or mutants makes it difficult to assess the function of Cdh2 in individual neurons at later stages of CNS development. In zebrafish, transplant experiments to assess *cdh2* mutant cell behavior in a wild-type host environment are difficult since *cdh2*-deficient cells do not integrate into ventral neural tube positions due to adhesive differences (Hong and Brewster, 2006; Lele et al., 2002). In this study, we bypass these problems by disrupting Cadherin-2 function specifically in FBMNs via expression of a dominant-negative form of Cadherin-2 under control of *islet1* enhancer elements that drive expression in cranial branchiomotor neurons. As our transgene was not expressed in the

surrounding neuroepithelial cells, we were able to examine the cell autonomous role of Cadherin-2 in FBMN migration without any confounding issues of neural tube morphogenesis. We found that inactivation of Cdh2 in FBMNs leads to defective caudal migration. Having several lines of stable transgenic fish (*vc23*, *vc25*) that displayed similar FBMN migration defects suggests that our observed phenotypes are due to functional Cdh2 inactivation and not positional effects of transgene insertion. The difference in phenotype severity across our lines underlies the importance of strong transgene expression when using a dominant-negative approach and indicates that partial defects in caudal migration, such as those seen in hemizygous *Tg(isl1:cdh2ΔEC-mCherry)* embryos represent a hypomorphic phenotype due to incomplete inactivation of Cdh2 function. In homozygous *vc25Tg* embryos, our highest expressing line, lack of Cdh2 function does not affect cell motility per se, but does result in a lack of directionality in FBMN cell soma movements. In the absence of sustained caudally directed migration, FBMNs often make ectopic apical and dorsal movements between neuroepithelial cells within r4 in *Tg(isl1:cdh2ΔEC-mCherry)* fish. It was previously suggested that FBMNs are excluded from entering the dorsal hindbrain due to neuroepithelial cell cohesion that stems from Cdh2-mediated homotypic neuroepithelial cell adhesion that ensures that FBMNs migrate along the ventral aspect of the hindbrain (Stockinger et al., 2011). Our observation that dominant-negative Cdh2-expressing FBMNs move dorsally despite normal Cdh2 function in neuroepithelial cells suggests that Cdh2-mediated neuroepithelial cell cohesion is not responsible for excluding FBMNs from the dorsal neuroepithelium. Interestingly, similar ectopic dorsally positioned FBMNs that fail to migrate caudally have been observed in embryos with a mutation in the PCP component Prickle1b (*pk1b*^{fh122}), where the surrounding neuroepithelial cells are planar polarized with normal structural integrity (Mapp et al., 2010; Mapp et al., 2011; Rohrschneider et al., 2007; Walsh et al., 2011). Thus, in both cases, inactivation of Cdh2 and depletion of Pk1b, FBMNs migrate randomly and dorsally even

when Cdh2-based neuroepithelial cell cohesion is unaffected. Ectopic dorsal positioning of FBMNs in the absence of Cdh2 function appears to be a consequence of a loss of directionality, and contributes to the overall defect in caudal migration. We are, however, unable to rule out the possibility that Cdh2-based neuroepithelial cell-to-FBMN heterotypic cell interactions provide a molecular signal to prevent ectopic apico-dorsal cell movement of FBMNs. Taken together, our findings suggest that Cdh2 acts cell autonomously to promote directional migration of FBMNs.

Despite a reduction in sustained caudal migration when Cdh2 is inactivated, we observed a small proportion of FBMNs that migrated out of r4 into r5 before stalling; a result previously reported in *cdh2* mutants and morphants (Stockinger et al., 2011; Wanner and Prince, 2013b). Our results clarify that the limited caudal migration into r5 is not a byproduct of defective neural tube morphogenesis, but rather suggests that FBMNs retain a limited capacity for caudal migration in the absence of Cdh2 function. Previous studies occasionally observed a small number of FBMNs ('escapers') that migrated into r6 in *cdh2* mutants and morphants (Stockinger et al., 2011; Wanner and Prince, 2013b). Similarly, we infrequently found escapers in r6 in some homozygous *vc25Tg* embryos. Our observation that 59% of homozygous *vc25Tg* embryos have at least one escaper neuron agree well with previous estimates of escapers in *cdh2* morphants (61.5% of embryos)(Wanner and Prince, 2013b). These authors described the leading or pioneer FBMN as having a crucial role in promoting the caudal migration of later-born follower neurons, at least into r5, since laser ablation of the pioneer neuron or its trailing axon impairs follower FBMN migration (Wanner and Prince, 2013b). These authors attributed the presence of escaper neurons in r6 of Cdh2-depleted embryos as pioneer neurons that migrate in a Cdh2-independent manner, suggesting that pioneer neurons have intrinsically different migratory properties than follower neurons. However, our observation that most *vc25Tg* embryos (86%) have either no escapers (41%) or escapers on only one side of the

embryo (45%) is not consistent with a model in which each embryo should have at least one pioneer neuron (per hemi-segment) that navigates caudally without the need for Cdh2 function. We suggest that Cdh2 is one of multiple guidance cues that FBMNs utilize to undergo sustained directed caudal migration. Thus, both leader and follower FBMNs must integrate Cdh2 function with other external guidance cues for robust caudal migration. Elimination of one guidance mechanism, such as Cdh2 neuron-to-neuron interactions, severely limits the overall effectiveness of caudal migration. Interestingly, partial migration phenotypes and 'escaper' neurons are not observed in embryos with mutations in PCP components(Bingham et al., 2002; Jessen et al., 2002; Pan et al., 2014; Rohrschneider et al., 2007; Wada et al., 2005; Wada et al., 2006b), in which FBMNs completely fail to exit r4, despite the observation that PCP mutant FBMNs are motile and migrate randomly within r4 (Jessen et al., 2002; Mapp et al., 2010). This suggests that Cdh2-deficient FBMNs may rely on PCP-dependent migration to undergo limited and sparing migration out of r4.

Cadherin-2 controls collective migration

FBMNs exhibit two modes of migration. First, FBMN migration involves interactions between migrating neurons and the surrounding neuroepithelium, an interaction that requires the function of PCP components both within FBMNs (cell-autonomously) and in the surrounding neuroepithelial cells (non cell-autonomously) (Davey et al., 2016; Walsh et al., 2011). For instance, wild-type FBMNs fail to migrate through a PCP-deficient neuroepithelial environment (Jessen et al., 2002; Wada et al., 2005; Wada et al., 2006b). On the other hand, when all FBMNs are PCP-deficient, they are unable to migrate caudally even when the environment is wild-type (Davey et al., 2016; Rohrschneider et al., 2007; Walsh et al., 2011). Second, FBMNs engage in a collective cell migration that involves FBMN-to-FBMN interactions. In chimeric embryos generated by cell transplantation, a wild-type FBMN can induce caudal directional movements from a neighboring PCP-deficient FBMN (Walsh et al., 2011). This influence of a

wild-type FBMN on the directional migration of another neuron characterizes this as a collective cell migration that involves neuron-to-neuron interactions but does not require cell autonomous PCP function in the rescued neuron. In previous studies assessing migratory phenotypes in chimeras, up to 40% of PCP-deficient neurons migrate caudally if adjacent to neighboring wild-type FBMNs (Walsh et al., 2011). It is striking then that dominant-negative Cdh2-expressing FBMNs completely fail to be “rescued” by neighboring wild-type FBMNs in mosaic embryos in this study. Our observations are consistent with a model in which Cdh2-mediated homotypic FBMN-to-FBMN cell interactions are the basis for collective cell migration in FBMNs.

What is the nature of the Cdh2-based neuron-to-neuron cell contact that drives collective cell behavior in FBMNs? As described above, the first FBMN to exit r4 represents the pioneer neuron that trails its axon behind it as it migrates caudally (Wanner and Prince, 2013b). Follower FBMNs are proposed to migrate using the pioneer axon as a substrate for adhesion and migration (Wanner and Prince, 2013b). In this scenario, Cdh2 may function to control attachment of follower neurons to pioneer axons and promote caudal migration along a preferred substrate. This could explain how PCP-deficient cells are ‘rescued’ by wild-type FBMNs, since ‘follower’ PCP-mutant FBMNs could still make use of Cdh2-mediated adhesion to migrate along the wild-type pioneer axon substrate to move in the caudal direction. In this case, cell-cell interactions would represent soma-to-axon adhesions between follower and pioneer FBMNs. This is reminiscent of a role for Cadherin-2 in radial glial fiber-dependent migration of cortical neurons, where Cdh2 mediates tight attachment of locomoting neurons and radial glial fibers (Kawauchi et al., 2010; Shikanai et al., 2011). An alternative possibility is that caudal directionality is obtained through Cdh2-based soma-to-soma cell contact between FBMNs. In this scenario, Cdh2-mediated cell contact is required for movement and that cell polarity and directionality are acquired as a consequence of soma-to-soma contact. Both

neural crest cells and mesendodermal cells migrate as single cells that utilize transient Cdh2-based cell-to-cell contacts to coordinate directionality of movement (Dumortier et al., 2012b; Richardson et al., 2016; Theveneau and Mayor, 2012b). Future studies using high-resolution time-lapse imaging will help resolve the precise nature of Cdh2-mediated neuron-to-neuron interactions that drive collective FBMN migration.

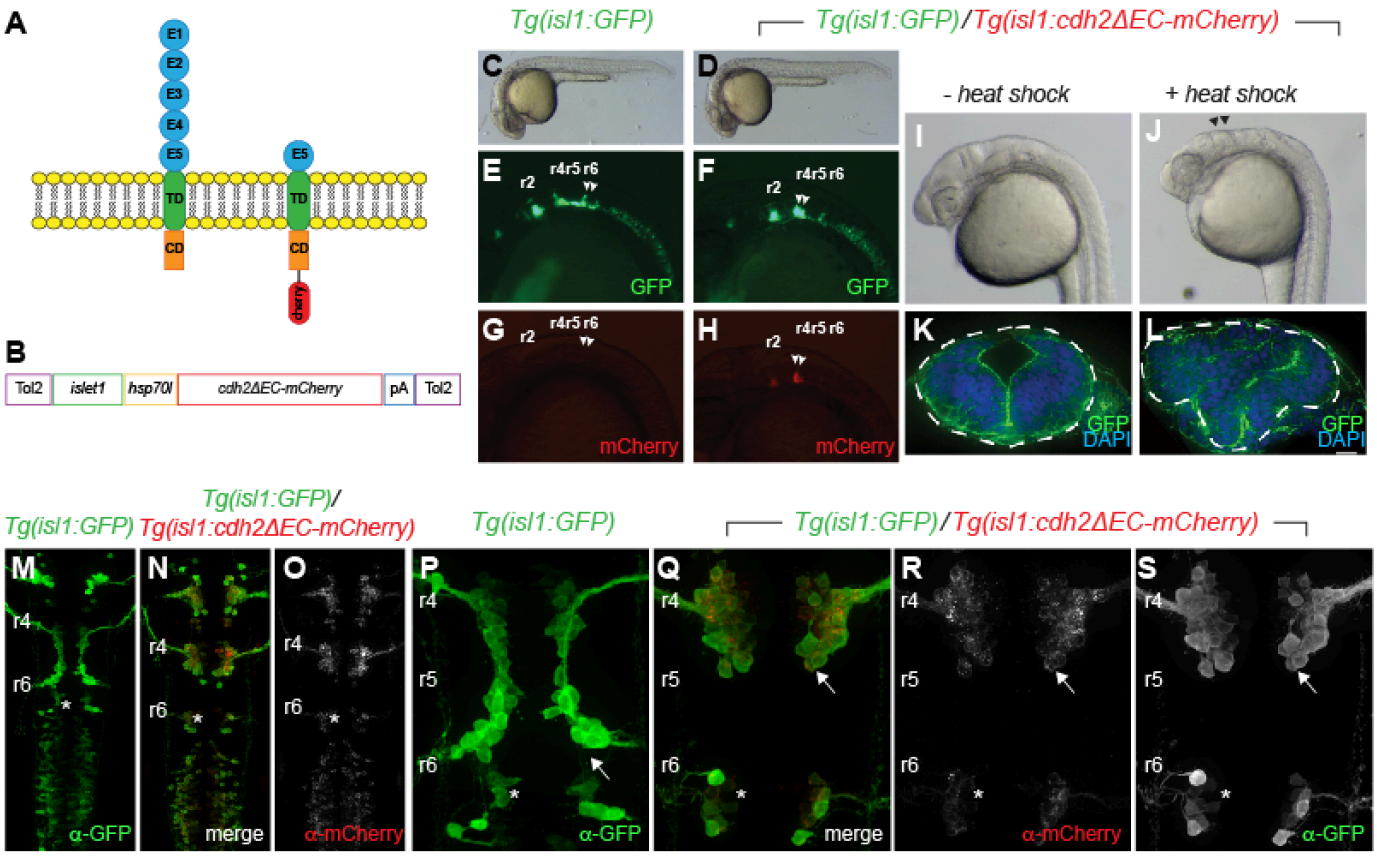

Fig. 2. Generation of stable transgenic fish expressing dominant-negative Cadherin-2 in cranial branchiomotor neurons. (A) Schematic representation of full length Cadherin-2 (N-cadherin) and extracellular domain-deleted Cadherin-2 (Cdh2ΔEC) fused with mCherry that functions as a dominant-negative protein. E1-E5 cadherin ectodomains; TD, transmembrane domain; CD, cytoplasmic domain. (B). Schematic representation of plasmid used to generate stable transgenic fish expressing *cdh2ΔEC-mCherry* driven by the *zCrest1* enhancer element of the *islet1* promoter (*islet1*) upstream of the minimal promoter (*hsp70l*). (C,D) Photographs of wild-type *Tg(isl1:GFP)* and *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos at 24hpf, showing normal morphology. (E-H) Lateral images at 24 hpf of the green and red channels showing GFP-expressing and Cdh2ΔEC-mCherry-expressing cranial branchiomotor neurons in *Tg(isl1:GFP)* fish and *Tg(isl1:cdh2ΔEC-mCherry)* transgenic fish. Arrowheads point to FBMNs. (I,J) Lateral images of *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos at 24hpf with and without heat shock. Arrowheads denote defects at midbrain-hindbrain region after heat shock. (K,L) Cross sections through hindbrain neuroepithelium of *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos before and after heat shock labeled with DAPI and Alexa-488-phalliodin. Dotted lines outline the neural tube. (M-S) Confocal micrographs of immunostained embryos showing low-magnification (M-O) and high magnification (P-S) dorsal views of wild-type *Tg(isl1:GFP)* (M,P) and *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos (N,O,Q-S) at 26 hpf. Embryos were labeled with α-GFP and α-mCherry showing that Cdh2ΔEC-mCherry is only expressed in cranial branchiomotor neurons and not the surrounding neuroepithelium. Arrow points to the abnormal position of FBMNs in *Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos. Rhomobomeres (r2-r6) are indicated. White asterisk denotes r6-derived PLL efferent neurons, which differ from r4-derived FBMN populations.

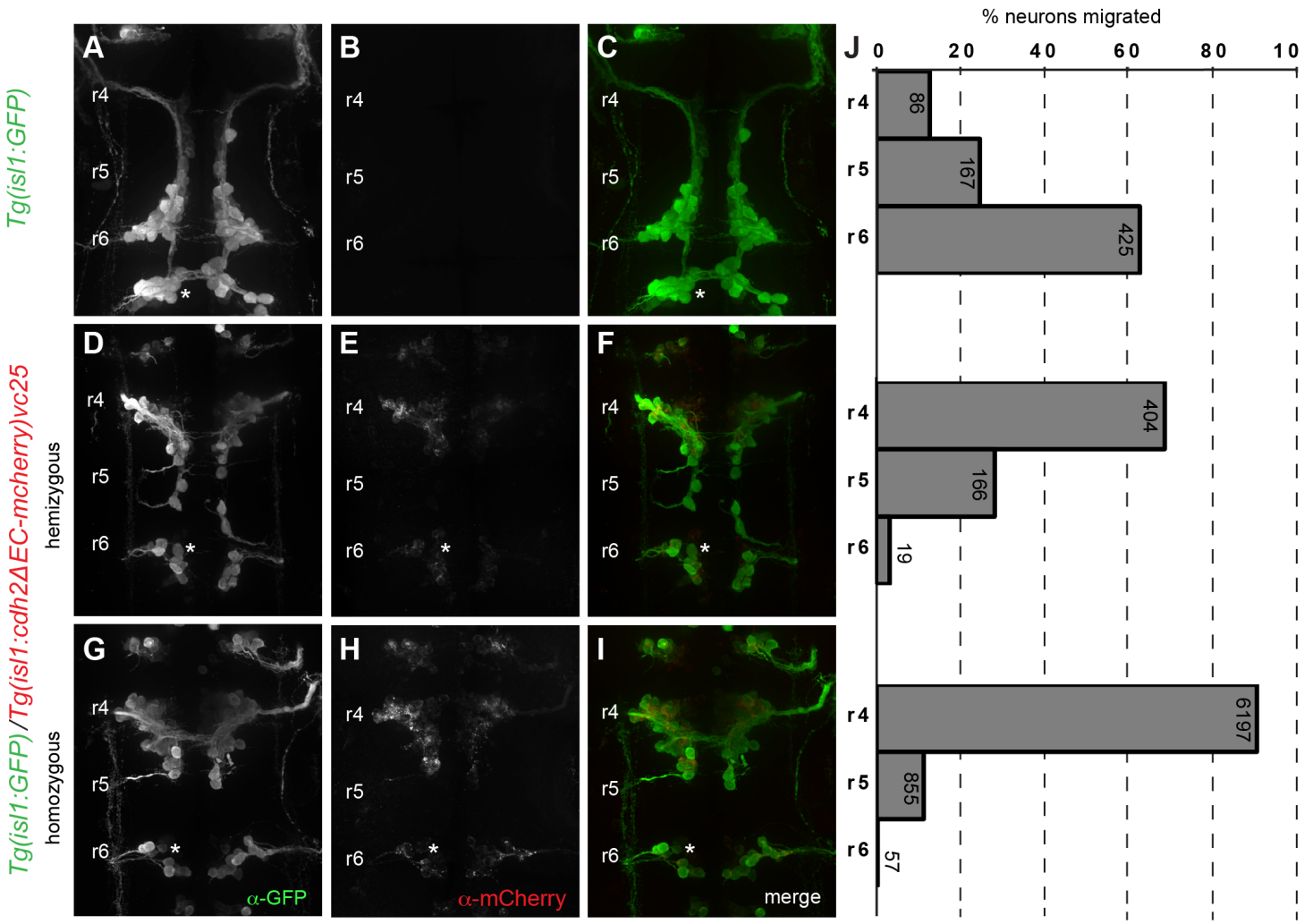

Fig. 3. Cadherin-2 is required cell autonomously for caudal migration of FBMNs. (A-I) Whole-mount immunocytochemistry showing dorsal views of *Tg(isl1:GFP)* (A-C) and *Tg(isl1:cdh2ΔEC-mCherry)vc25* transgenic embryos (D-I) at 38 hpf embryos. Embryos are labeled with α-GFP (green) (A,D,G) and α-mCherry (red) (B,E,H) antibodies. (A-C) Wild-type *Tg(isl1:GFP)* embryos with FBMNs fully migrated into r6. (D-I) Defective caudal migration of FBMNs in *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos carrying one copy of the transgene (hemizygous) or two copies (homozygous). (J) Histograms indicate the percent of FBMNS at 38 hpf that failed to migrate (r4), migrated partially (r5), or migrated fully (r6). Each histogram corresponds to the genetic condition in the image to its left and numbers indicate the number of FBMNs counted. White asterisk denotes r6-derived PLL efferent neurons, which differ from r4-derived FBMN populations.

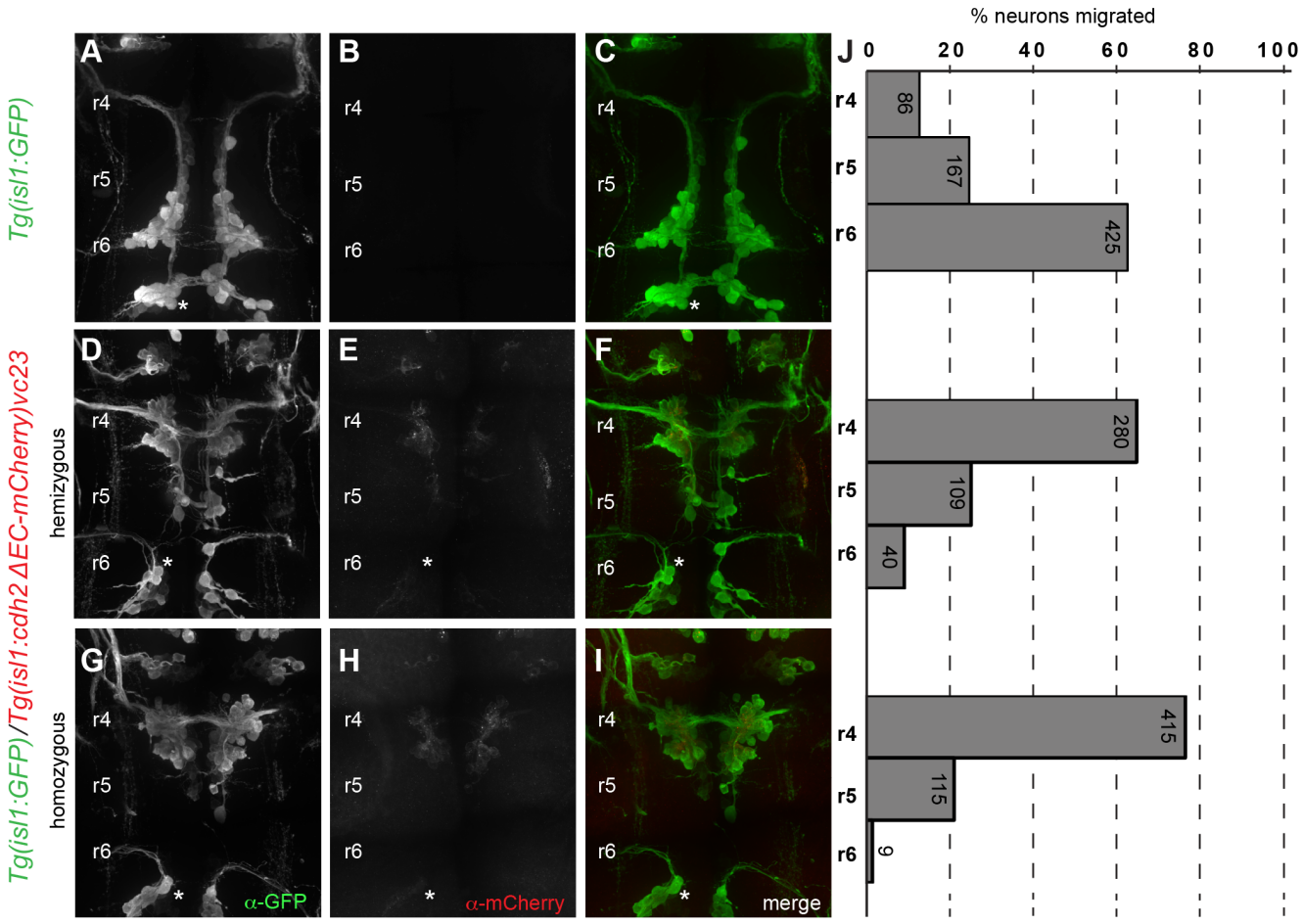

Fig. 4. Cadherin-2 is required cell autonomously for caudal migration of FBMNs. (A-I) Whole-mount immunocytochemistry showing dorsal views of *Tg(isl1:GFP)* (A-C) and *Tg(isl1:cdh2ΔEC-mCherry)vc23* transgenic embryos (D-I) at 38 hpf embryos. Embryos are labeled with α-GFP (green) (A,D,G) and α-mCherry (red) (B,E,H) antibodies. (A-C) Wild-type *Tg(isl1:GFP)* embryos with FBMNs fully migrated into r6. (D-I) Defective caudal migration of FBMNs in *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc23* embryos carrying one copy of the transgene (hemizygous) or two copies (homozygous). (J) Histograms indicate the percent of FBMNS at 38 hpf that failed to migrate (r4), migrated partially (r5), or migrated fully (r6). Each histogram corresponds to the genetic condition in the image to its left and numbers indicate the number of FBMNs counted.

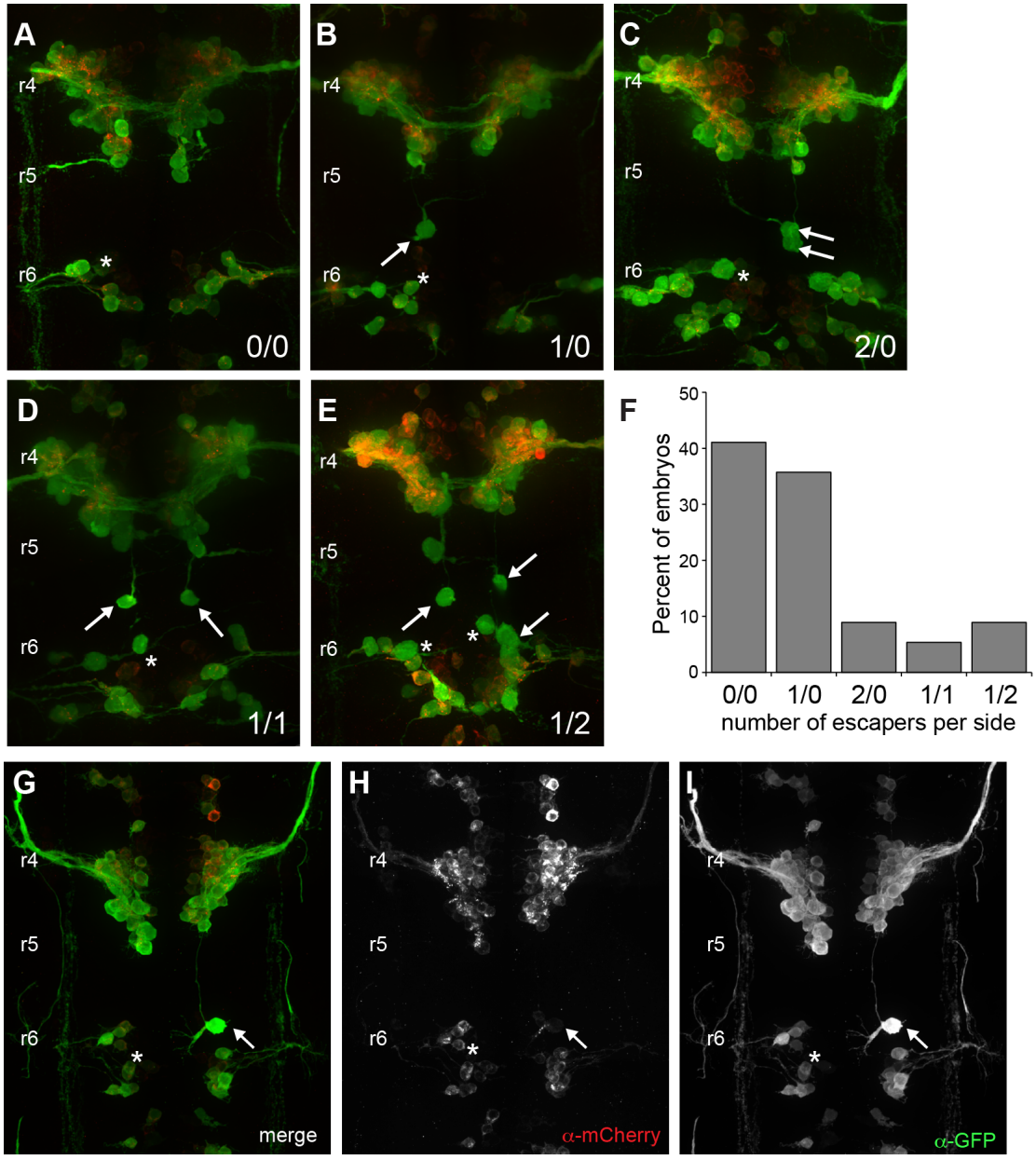

Fig. 5. Migration of 'escaper' neurons in homozygous *Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos. (A-E) Confocal micrographs of dorsal views of homozygous *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos at 38 hpf. Embryos were labeled with α-GFP (green) and α-mCherry (red). Representative images of homozygous *vc25Tg* embryos that shows the majority of FBMNs fail to exit r4/r5 with or without a rare 'escaper' FBMN that migrates into r6 (arrows). (A) An embryo with no 'escaper' neurons present in r6 on either side of the midline (0/0). (B). An embryo with one 'escaper' neuron present in r6 on one side of the embryo, with no 'escapers' on the contralateral side (1/0). (C) An embryo with two 'escaper' FBMNs present in r6 on one side of the embryo and no 'escapers' on the contralateral side (2/0). (D) An embryo with one 'escaper' neuron present in r6 on both sides of the embryo (1/1). (E) An embryo with one 'escaper' neuron present in r6 on one side of the embryo and two 'escaper' FBMNs present on the contralateral side (1/2). (F) Histogram reflects the percentage of homozygous *Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos with each 'escaper' condition. (G-I) Confocal micrographs of immunostained embryos showing high magnification dorsal views of *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryo at 38 hpf. White arrow shows 'escaper' neuron that expresses both *isl1:GFP* (green) and *isl1:cdh2ΔEC-mCherry* (red)

transgenes, despite its presence in r6. White asterisk denotes r6-derived PLL efferent neurons, which differ from r4-derived FBMN populations.

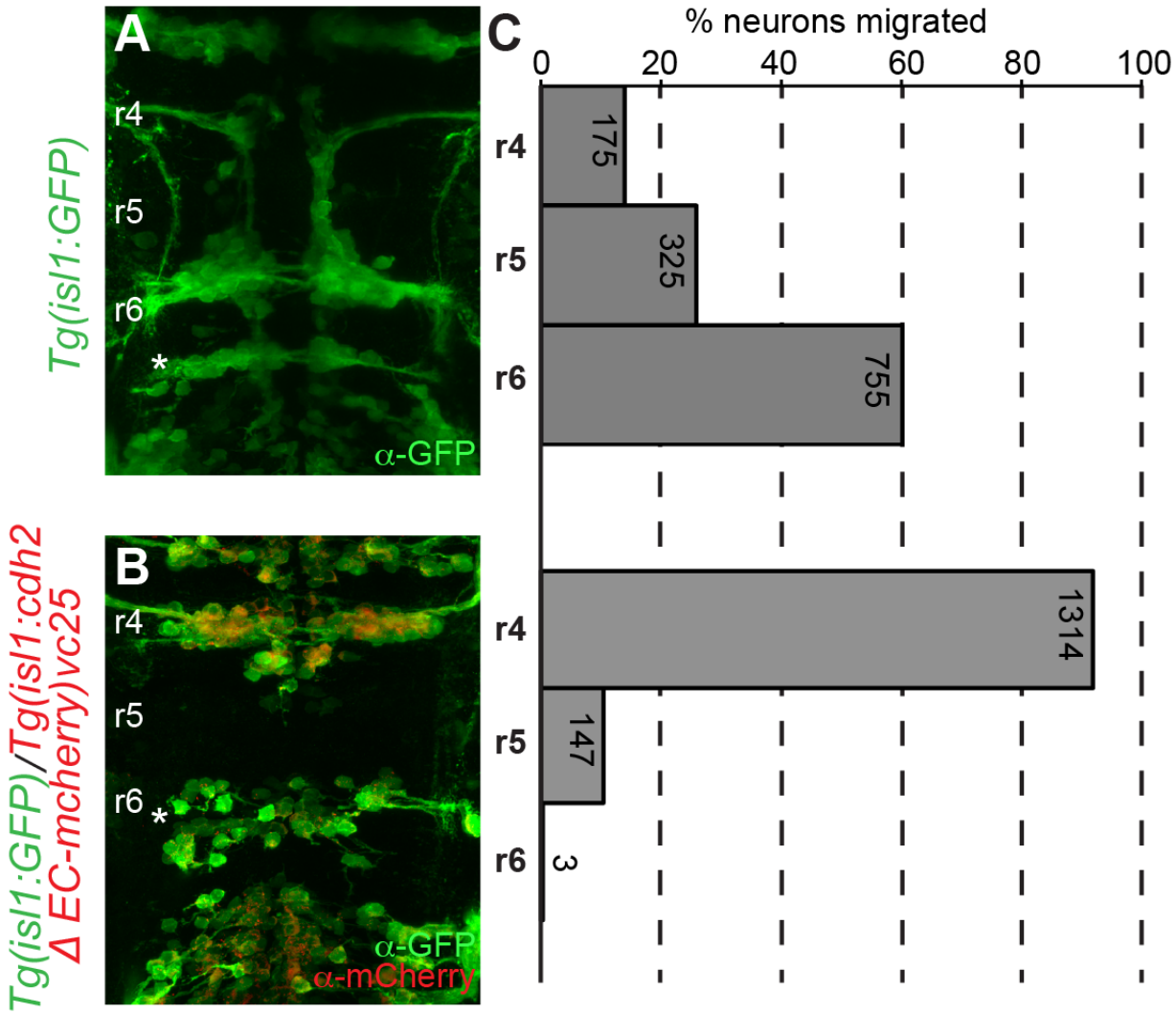

Fig. 6. Defect in caudal migration is not due to a delay in cell movements in *Tg(isl1:cdh2ΔEC-mCherry)vc25* transgenic embryos. (A-B) Whole-mount immunocytochemistry showing dorsal view of wild-type *Tg(isl1:GFP)* (A) and *Tg(isl1:cdh2ΔEC-mCherry)vc25* transgenic embryos (B) at 48 hpf. Embryos are labeled with α-GFP (green) (A and B) and α-mCherry (red) (B) antibodies. (A) Wild-type *Tg(isl1:GFP)* embryos with FBMNs fully migrated into r6. (B) There is a dramatic defect in caudal migration of FBMNs in homozygous *Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos at 48 hpf, when FBMN migration is normally complete. (C) Histogram indicates the percent of FBMNS at 48 hpf that failed to migrate (r4), migrated partially (r5), or migrated fully (r6). Each histogram corresponds to the genetic condition in the image to its left and numbers indicate the number of FBMNs counted. White asterisk denotes r6-derived PLL efferent neurons, which differ from r4-derived FBMN populations.

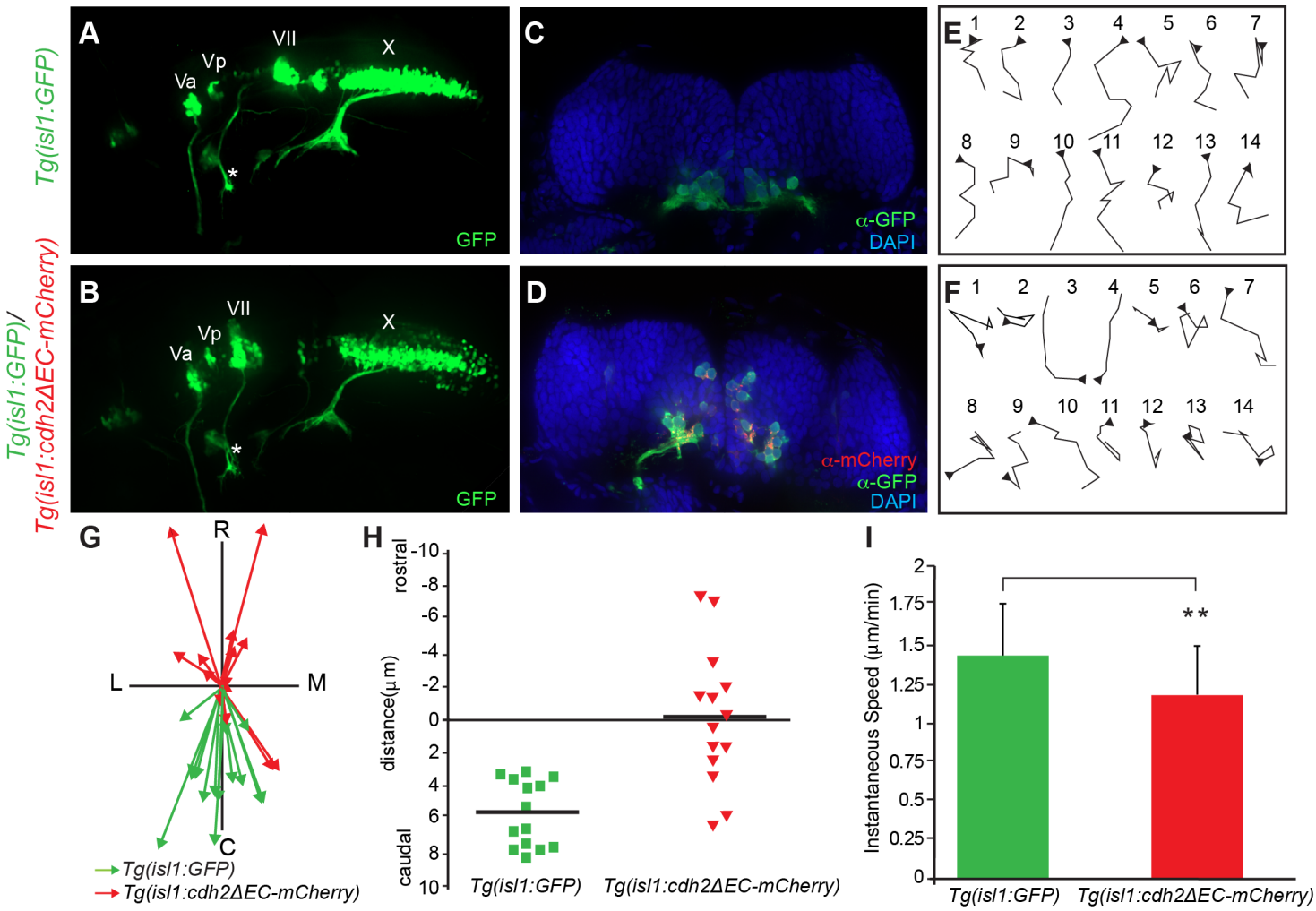

Fig. 7. Inactivation of Cadherin-2 affects directionality of FBMN migration (A,B) Live imaging of lateral views of the hindbrain showing the positioning of CBMNs and their peripheral axonal projections at 48 hpf. (A) In wild-type *Tg(isl1:GFP)* embryos, FBMNs (VII) migration is complete and their axons (asterisk) project into the second branchial arch. (B) In T*g(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos, the facial axons (asterisk) and trigeminal (Va,Vp), and vagus (X) axons can be seen to project normally. However, the FBMNs (VII) remain in r4 and lie in an abnormal dorsal position. (C,D) Coronal sections of 48hpf hindbrain from *Tg(isl1:GFP)* embryos at the level of r6 and *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos at the level of r4. FBMNs (α-GFP, green) in control *Tg(isl1:GFP)* embryos occupy a ventral position within r6. Cdh2ΔEC-mCherry-expressing FBMNs (α-mCherry, red) are found ectopically in a dorsal portion within r4. Nuclei are labeled with DAPI (blue). (E,F) Tracings of migratory paths of FBMNs captured from time-lapse images between 20-24 hpf from *Tg(isl1:GFP)* and *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos. Each time-lapse lasted 35 minutes with one frame every 5 minutes. Each trace is oriented so that caudal is to the bottom and medial is to the right. Arrowheads indicate the starting point for each cell. (G) Plot of the migratory tracks from start to endpoint shows a highly directional caudal migration of wild-type FBMNs (green arrows) in comparison to the random paths taken by Cdh2ΔEC-mCherry-expressing FBMNs (red arrows). C, caudal; R, rostral; L, lateral; M, medial (H) Quantitation of average distance traveled along the rostral-caudal axis by FBMNs in *Tg(isl1:GFP)* and *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos during the time-lapse sequences. (I) Quantitation of average instantaneous speed of FBMN movements in

Tg(isl1:GFP) and *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryos. (Mean values ± SD are shown; $p < 0.05$).

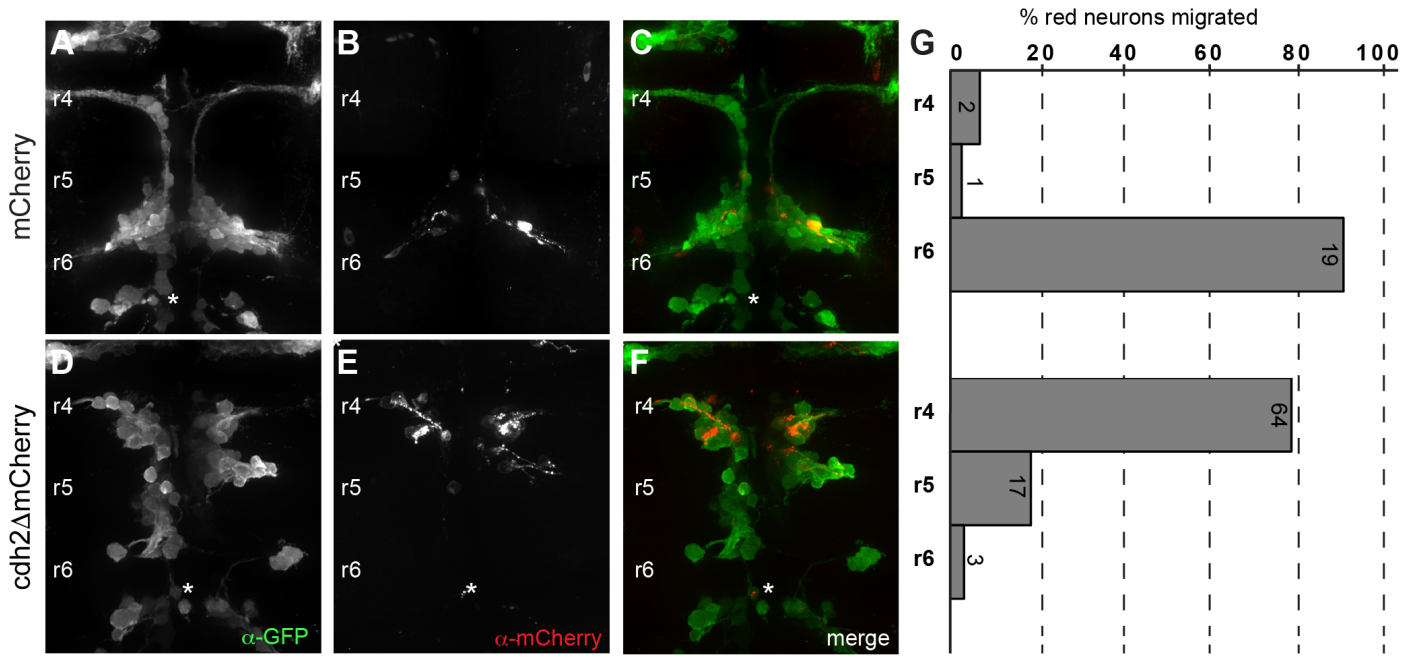

Fig. 8. Cadherin-2 is required for collective migration of FBMNs. (A-C) Confocal images showing dorsal views of *Tg(isl1:GFP)* embryos at 38 hpf injected with plasmids driving expression of mCherry alone or Cdh2ΔEC-mCherry mosaically in CBMNs. Embryos are labeled with α-GFP (green) and α-mCherry (red). Expression of mCherry alone has no effect on the caudal migration of FBMNs. (D-F) In contrast, FBMNs expressing Cdh2ΔEC-mCherry do not migrate caudally even though neighboring wild-type FBMNs that do not express the transgene migrate appropriately towards r6. (G) Quantitation of the percent of mCherry- or Cdh2ΔEC-mCherry-expressing FBMNs that failed to migrate (r4), migrated partially (r5), or migrated fully (r6). Each histogram corresponds to the condition in the image to its left and numbers indicate the number of FBMNs counted. White asterisk denotes PLL efferent neurons, which differ from r4-derived FBMN populations.

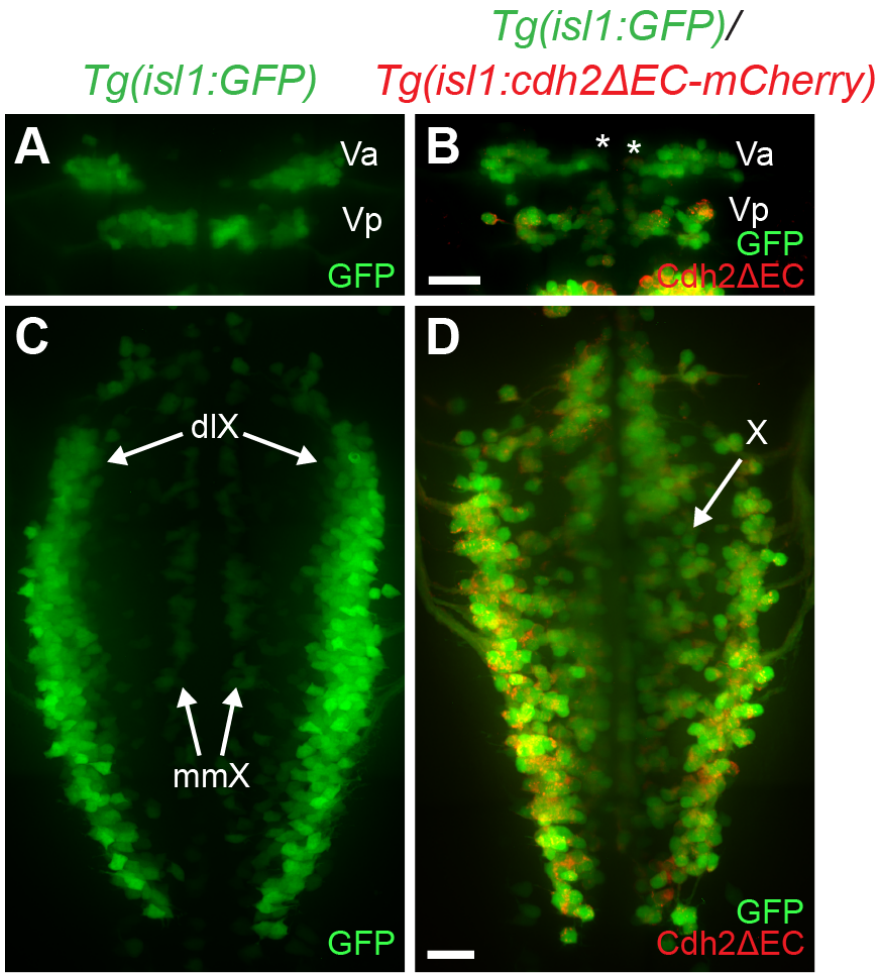

Fig. 9. Expression of dominant-negative Cadherin-2 in trigeminal and vagus branchiomotor neurons leads to aberrant neuron positioning. (A) Dorsal view of live *Tg(isl1:GFP)* embryo at 48hpf shows positioning of anterior and posterior clusters of trigeminal neurons (Va,Vp) found in r2 and r3, respectively. Note the lateral positioning of the Va cluster of trigeminal motor neurons at 48 hpf. (B) Dorsal view of live *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryo at 48 hpf shows that trigeminal neurons (Va; asterisk) remain in a medial location. *Green* is the GFP signal, whereas *red* is the Cdh2ΔEC-mCherry signal. (C) Dorsal view of live *Tg(isl1:GFP)* embryo at 48hpf shows correct positioning of vagus motor neurons in dorsolateral motor nucleus (dlX) and medial motor nucleus (mmX). (D) Dorsal view of live *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc25* embryo shows that vagus neurons (X) do not migrate and coalesce into discrete dorsolateral nuclei. Scale Bars = 20 μm.

Chapter 3: FBMNs use multiple cues to migrate collectively

Introduction

Neuronal migration is a fundamental step in neurodevelopment. Many neurons must travel variable distances from their place of birth to where they become integrated into a functional circuit. There are many mechanisms neurons utilize in order to achieve this migration and mutations in genes that regulate cell migration have been shown to cause rare genetic disorders such as schizophrenia, autism, tourette's syndrome epilepsy and mental retardation (Ross and Walsh, 2001; Valiente and Marín, 2010; Wanner et al., 2013). As previously established by cell transplant experiments have suggested FBMNs use collective migration in order to migrate properly from r4 to r6 (Walsh et al., 2011).

Collective cell migration, by definition, is the coordinated migration of cells as tight clusters or loose groups such that cooperation between cells contributes to the overall directionality of the cells (Theveneau and Mayor, 2012a). Collective migration has been extensively studied in tissue culture in vitro and neural crest cells in vivo, but there is little known about the distinct cellular mechanisms FBMNs use to migrate collectively. We previously established a model for collective migration in neural development where collective cell behaviors of FBMNs are driven by Cdh2-mediated neuron-neuron interactions. It is still unclear how neuron-neuron interactions influence FBMN collectively. Thus, we looked to other cell types to see how cell-cell interactions influence migration. In collective migration epithelial cells, stable cell-cell interactions are required to keep sheets of cells physically tethered to one another for collective migration of the entire group (Rørth, 2009). In neural crest cells, groups of cells migrate together in loose streams and make transient cell-cell contacts to restrict protrusive activity to the free space, a phenomena referred to as contact inhibition of locomotion (CIL). CIL ensures individual cells do not form protrusions between each other, do

not pile on tope one another and restricts migration of the collective group to the desired target tissue (Rørth, 2009).

Traditionally, CIL has been characterized as a mechanism of dispersion required for many different cell types. Initially, almost 70 years ago, influential cell biologist, Michael Abercrombie who studied the social behavior of cells in chick fibroblast explants, in vitro, characterized CIL. Abercrombie and colleagues coined the term CIL as a phenomena where colliding cells halted migration, collapsed protrusions, reformed protrusions into free space and migrated away from each other (Abercrombie, 1979). Since then, CIL has been studied and characterized as essential to directional migration in variety of cell types *in vivo*. Such *in vivo* studies include *Xenopus* neural crest cells, *Drosophila* macrophages (hemocytes) and Cajal-Retzius neurons in the cortex of mouse models (Carmona-Fontaine et al., 2008; Stramer et al., 2010; Villar-Cerviño et al., 2013). Hemocytes and Cajal-Retzius cells use CIL as mechanisms of directional dispersion during development for proper adult immune and neural functioning. In neural crest cells, however, CIL is required for proper directive migration of individual cells to ensure they invade proper tissue collectively. Thus it is of question to explore the possibility that neurons could use CIL to collectively migrate to their final destinations.

In addition to CIL, many migratory cell types must integrate multiple guidance cues, some attractive, some repulsive, to navigate to their correct destination. The ability for cells to receive and amalgamate these sometimes-conflicting cues together is essential during several developmental processes. For example, during visual system development, retinal ganglion cells are exposed to attractive surface bound ephrins on surrounding cells and a repulsive soluble Wnts in their environment (Schmitt et al., 2006). Additionally, neural crest cells balance attractive cues from soluble chemokine Sdf1 and repulsive cues caused by cell-cell contact (CIL) (Carmona-Fontaine et al., 2008; Theveneau and Mayor, 2010). Similar to these cell

types, we know that neurons are exposed to multiple cues as well during their directional migration, but it is still unclear how they are able to integrate these sometimes opposing cues.

FBMNs are an ideal model system to study collective migration in the nervous system. FBMNs are a subset of cranial motor neurons in the ventral hindbrain which are born in rhombomere 4 (r4) and migrate caudally into r6 (Chandrasekhar, 2004). We know from previous cell-transplant experiments, FBMNs are able to migrate collectively because the migration of one neuron influences the migration of neighboring neurons(Walsh et al., 2011). It has been proposed in other studies that physical interactions between FBMNs and the pioneer neuron's axon is required for proper caudal migration (Wanner and Prince, 2013a). However, we know this collective migration is mediated through Cdh2 cell-cell contact, as neurons deficient in Cdh2 signaling are unable to be influenced by neighboring neurons, regardless of then presence of a pioneer axon(Rebman et al., 2016). The nature of these physical interactions that mediate collectiveness and how FBMNs specifically integrate contact-mediated cues with other external cues is still unclear.

In this study, we examined the nature of physical interactions in FBMNs during migration. Expression of membrane-bound GFP allowed us to view cranial motor neurons and their processes during migration in order to examine physical interactions. We observed FBMNs migrate in close proximity to each other, frequently interacting with each other as opposed to an axon. Using live imaging, we also observed that FBMNs appear to participate in CIL during migration. Additionally, consistent with other cell types and past studies in the nervous system, we showed impaired caudal migration when we knocked down the function of chemokine system Sdf1/cxcr4b, suggesting FBMNs are influenced by multiple cues during migration. Taken together, these results are consistent with a model that FBMNs migrate collectively integrating CIL and chemotaxis together.

Materials and Methods

Zebrafish Husbandry

Zebrafish (*Danio rerio*) were raised following standard procedures and used in accordance with protocols approved by the VCU Institutional Animal Care and Use Committee. All zebrafish used in this study were maintained according to standard procedures(Westerfield, 2000) and staged as previously described(Kimmel et al., 1995).All mutant lines were used previously: *cxcr4bt26035 contains a nonsense mutation that results in a premature stop at codon 239* (Knaut et al., 2002) Transgenic fish carrying the *islet1* promoter driving GFP (*Tg(isl1:GFP)rw0*; Higashikima et al., 2000) have been described previously. Transgenic fish using the islet1 promoter to drive expression of a dominant-negative Cadherin-2 (Cdh2) that lacks a portion of the extracellular domain has been described previously (*Tg(isl1:cdh2deltaEC)*; (Rebman et al., 2016). Transgenic fish carrying the islet1 promoter driving GFP-CaaX expression [*Tg(islet1:GFP-CAAX)vc29*] [*Tg(islet1:GFP-CAAX)vc33*] were generated in this study.

Plasmid DNA Constructs

The *zCrest1* enhancer of the islet-1(isl1) regulatory elements along with the minimal promoter from the heat shock protein 70, like (hsp70l) were PCR amplified, blunted and cloned into the EcoRV site in pTolDest (gift of Dr. Nathan Lawson) to make pTol-isl1-hsp70l-DEST. For simplicity, we will refer to the *isl1-hsp70l* promoter hereafter as the *isl1* promoter EGFP-CaaX, located in pME-GFP-CaaX, was cloned into pTol-isl1-Dest vector, using Gateway cloning (Thermofisher) to generate pTol-isl1:GFP-CaaX-Dest.

Generation of *Tg(isl1:GFP-CaaX)* transgenic lines

Capped mRNA for Tol2 *transposase (reference Kawakami)* was in vitro transcribed using the mMESSAGE mMachine kit (Ambion). DNA encoding each plasmid (50 ng/uL) was co-injected with Tol2 *transposase* mRNA (50 ng/uL) into AB embryos at the 1 cell stage.

Founder (F0) embryos were screened for mosaic GFP expression in cranial motorneurons at 24 and 48 hpf. F0 embryos, displaying GFP expression in cranial motorneurons, were raised to adulthood. Germline transgenic founders were identified by screening F1 progeny for GFP fluorescence. Several founders with specific expression in cranial motorneurons, and little to no off-target expression, were isolated, and their GFP-positive progeny were raised to adulthood. Two high expressing F1 lines *Tg(isl1:GFP-CaaX)vc29* and was *Tg(isl1:GFP-CaaX)vc33* were outcrossed and F2 generations were made.

Morpholino injections

Antisense morpholinos (MO) were injected at the 1-cell stage. Morpholinos were co-injected as follows: Sdf1a: (5'-ATCACTTTGAGATCCATGTTGCA); 2ng (David et al., 2002); p53: (5'-GCGCCATTGCTTTGCAAGAATTG); 2ng.

Immunohistochemistry

The following primary antibodies were used. GFP: Mouse anti-GFP (DSHB ;1:100), Rabbit anti-GFP (Invitrogen; 1:500), Mouse anti-mCherry (NOVUS; 1:250). Embryos were manually dechorionated and fixed in 4% paraformaldehyde at 4°C overnight, washed in PBST (1×PBS with 0.25% Triton X-100), and incubated for one hour at room temperature in blocking solution (PBST + 10% Goat Serum + 4% BSA). Embryos were then incubated in the corresponding primary antibody and diluted in blocking solution at 4°C overnight. They were then washed in PBST and incubated overnight at 4°C in secondary antibodies. The following secondary antibodies were used at a concentration of 1:200: Alexa Fluor 488 Goat anti-mouse IgG (H+L) (A11029, Life Technologies), Alexa Fluor 488 Goat anti-Rabbit IgG (H+L) (A11034, Life Technologies). Alexa Fluor 568 Goat anti-mouse IgG (H+L) (A11031, Life Technologies). Alexa Fluor 568 Goat anti-rabbit IgG (H+L) (A11079, Life Technologies). Embryos were washed in PBST and sequentially dehydrated in 25%, 50%, and 75% glycerol in 1× PBS.

Yolks were removed using sharpened tungsten wire and embryos were flat mounted on coverslips and surrounded with 75% glycerol medium.

Microscopy and time lapse imaging

Confocal images of immunostained embryos were obtained on an inverted Zeiss Spinning Disk Laser Confocal Observer Z1 using a Zeiss Plan-Apochromat 63X/1.2 W objective, equipped with a Photometrics Evolve EMCCD camera for live time-lapse imaging. For timelapse live imaging, embryos were anesthetized in embryo medium containing tricaine, manually dechorionated and mounted in 1.2% low-melting point agarose on a glass bottom dish (Fluorodish; World Precision Instruments). Time-lapse imaging was performed at 28.5°C using a heated stage insert. For time-lapse imaging, z-stack images at 35 nm steps were captured every 5 minutes for up to 8 hours. The acquired z-stacks were exported and analyzed using AR-Elements (Nikon) software.

Statisical Analysis

Quantitation of cell positions and cell collision outcomes were done on a per embryo basis. Statisical significance was determined using unpaired t-test.

Results

Facial Branchiomotor neurons (FBMNs) use soma-soma contact during caudal migration

FBMNs interact physically with each other and their environment during caudal migration. Previous reports have suggested that these physical interactions are required for proper FBMN migration (Stockinger et al., 2011; Wanner and Prince, 2013), however, the nature of these physical interactions remains unclear. To begin to investigate the nature of neuron-neuron physical interactions that mediate collective movement of FBMNs, we first examined FBMNs at early stages of migration from r4 into r5. To accomplish this, we

generated stable transgenic zebrafish line that expresses membrane-bound green fluorescent protein (GFP-CaaX) specifically in cranial motor neurons under control of the *zcrest1* enhancer of the *islet1* promoter, using Tol2-mediated transgenesis (Kawakami, 2007)(Fig. 10 A-B). We then used immunostaining to examine the morphology and position of neurons at early time points in these *Tg(isl1:GFP-CaaX)* embryos (Fig. 11A-D). The most caudally positioned FBMN migrates out of r4 first (leader cell) trailing its axon behind it (Wanner and Prince, 2013a). It has previously been suggested that this axon is required for subsequent neurons to migrate out of r4. In 26 examined embryos, the vast majority of FBMNs migrated without making contact with the leader neuron's axon (79.7%, n=339neurons) when compared to those that did make contact with the leader neuron's axon (20.4%, n=339 neurons) Neurons were much more likely to make soma-soma contact each other (88%, n=339) neurons (Vareed and Walsh, personal communication). This finding shows that soma-soma interactions between neighbouring neurons, rather than soma-axon interactions, are the predominant site of neuron-neuron physical interactions. This observation suggests that soma-soma contact may be important in mediating the collective behaviour of FBMNs.

Facial Branchiomotor neurons (FBMNs) engage in CIL during development

We next sought to determine whether soma-soma cell contact influences the directional migration of FBMNs. In other cell types, such as cranial neural crest cells, collective migration has been shown to be orchestrated by Contact Inhibition of Locomotion (CIL) *in vivo* (Stramer and Mayor, 2016). CIL is a process by which cells make contact upon collision, a suppression of forward movement upon neuron-neuron contact, followed by a collapse of protrusions, and ultimately a change in migration direction. As FBMNs migrate in an anterior-to-posterior direction, following neurons are making soma-soma collisions with leading (more posterior) neurons. We therefore examined pairwise cell collisions between two neighboring FBMNs and classified the outcome as CIL or non-CIL (Fig. 12A). We would predict that if CIL occurs, a

given collision would result in the leader neuron moving posteriorly and the follower cell would be re-directed anteriorly post-collision (Figs. 12B, 12E, 12F). A non-CIL event occurs when, upon collision, both cells, leader and follower, continue in the posterior direction (Fig. 12C).

We began by recording high-resolution time-lapse images of FBMN migration *in vivo* in wild-type *Tg(isl1:GFP)* embryos. We recorded movies at one frame every 5 minutes between 18 and 26 hpf and quantified the outcome of cell collisions between migrating FBMNs. We focused our analysis on the first 8-10 cells that migrated posteriorly out of r4. We found numerous cell collisions resulting in repulsion of the trailing cell leading to a transient halt in migration, or more often a redirection of motility in the opposite direction (anteriorly). Following these collisions, the leading cell would resume or continue movement posteriorly. In fact, CIL outcomes accounted for 49 ± 2.64% of the cell collisions we recorded (n=93 collision events across 10 embryos) (Fig. 12D).

Since CIL is classically defined as a collision event followed by protrusive changes and finally a change in direction, we predicted that cell collision events would result in a collapse of protrusions at the site of contact, followed by protrusions forming on the opposite side of the cells and or into free space. To accomplish this, we visualized cell protrusions at high resolution in real time using our *Tg(isl1:GFP-CaaX)* embryos (as described above). Consistent with stereotypical CIL behaviour, we observed a rapid disassembly of protrusions at the site of neuron-neuron contact, followed by formation of protrusions on the opposite side of the contact event. (Fig. 13 A-H).

CIL is not sufficient for sustained caudal migration

Although we have suggested CIL as a mechanism used during FBMN migration, it is traditionally thought of as a dispersion mechanism in most cell types (Carmona-Fontaine et al., 2008; Stramer et al., 2010; Villar-Cerviño et al., 2013). Thus, CIL cannot be the only guidance cue FBMNs respond to promote persistent posterior migration. (Fig. 12D). There must be other

guidance mechanisms that allow FBMNs to overcome CIL behaviour in order to migrate appropriately into r6/r7.

Previous research has suggested that CIL repulsion can be overcome by chemotactic signalling in cancer cells *in vitro* signals (Lin et al., 2015). We therefore sought to determine whether chemotactic signals play a role in FBMN migration and whether chemotactic signalling can influence cell collision outcomes.

Previous studies have suggested that proper FBMN migration depends on stromal-derived factor 1a (Sdf1a/Cxcl12) signalling (Cubedo et al., 2009; Sapède et al., 2005). First, *sdf1a* is expressed in r4 to r6, with highest expression levels in r6. By 24 hpf, *sdf1a* expression becomes restricted to r4 and r6 (Cubedo et al., 2009; Lewellis et al., 2013; Zannino et al., 2012). Second, FBMNs express the Sdf1a receptor, Cxcr4b, prior to and during migration, and are the only cell types to express this receptor within r4-r6 territory (Cubedo et al., 2009; Sapède et al., 2005). To confirm that Sdf1a/Cxcr4b is required for proper FBMN migration, we performed loss-of-function experiments using *sdf1a* morpholino and *cxcr4b* mutants (Fig. 14A-D). In wild-type embryos, the vast majority of FBMNs migrate into r6 (64.5 ± 4.6% ; n=4) (Fig. 14A). In both *sdf1a* knockdown (35.7 ± 5.1;n=6) and *cxcr4b* mutants (30.3 ± 18.1; n=5), FBMN migration is partially impaired (Fig. 14C,D), with neurons becoming distributed between r4 and r6 (Fig. 14E), confirming previous reports (Sapede et al., 2005). There is a statistically significant decrease in the number of neurons that migrate into r6 in both *sdf1a* knockdown and *cxcr4b* mutants ($p<0.05$; t-test)

We next investigated whether CIL outcomes after neuron-neuron collisions would be affected by Sdf1/Cxcr4 signalling (Fig. 15 A-D). We performed live imaging of FBMNs in *Tg(isl1:GFP)* fish with and without *sdf1a* knockdown and quantified pairwise FBMN collisions as CIL or non-CIL. In *sdf1a*-depleted embryos, there was a significant increase in the proportion of collisions that favour CIL outcomes leading to the follower (anterior) neuron

halting and transiently migrating back toward r4 (70.4 ± 2.42%; n=4;$p < 0.05$) (Fig. 15 D). This suggests that the presence of Sdf1 signalling overrides CIL outcomes to promote a larger number of non-CIL outcomes, or posterior-posterior outcomes.

Integration of SDF1-chemotaxis and CIL in FBMN migration

We have previously shown that Cdh2 is required for the collective behaviour of FBMNs (Rebman et al., 2016). Interestingly, when Cdh2 is inactivated specifically in cranial motor neurons (in *Tg(isl1:cdh2ΔEC-mCherry)vc25* (vc25Tg) embryos), FBMNs almost completely fail to migrate posteriorly (Rebman et al., 2016), highlighting the essential role for Cdh2-mediated cell-cell interactions are for the initiation of FBMN migration.

To further explore how CIL functions with chemotaxis, we needed an intermediate condition where Cdh2 is only partially inactivated. This would allow some Cdh2 signalling for FBMNs to contact each other and likely participate in CIL. Therefore, we utilized *Tg(isl1:cdh2ΔEC-mCherry)vc23* (vc23Tg) hemizygous fish that have one copy of the dominant-negative Cdh2 transgene. Due to positional effects, these transgenic embryos exhibit lower level of transgene expression when compared to the *vc25Tg* line, leading to a less severe migration defect (see (Rebman et al., 2016) for further details). In *vc23Tg* embryos, FBMNs have a partial migration defect resulting in FBMNs in r4, r5, and r6 (Rebman et al., 2016). Additionally, as discussed above, *sdf1a* morphants also display a partial migration defect where neurons are distributed from r4 to r6.

In order to study the effect of chemotactic cues (Sdf1) and CIL (Cdh2) together, we injected *sdf1a* morpholino into *vc23Tg* hemizygous embryos, allowed them to develop until ~42hpf (a time point where migration is mostly complete) and quantified the position of FBMNs (Fig. 16 A-E). We found that partial Cdh2 inactivation coupled with Sdf1a-depletion led to a more severe defect in posterior migration of FBMNs (Fig. 16 D,E), with a majority of FBMNs unable to migrate out of r4 (72.5 ± 11.9%; n=8; $p < 0.05$; t-test) when compared to *vc23Tg*

embryos alone (38.4± 10.9%; n = 8; $p < 0.05$; t-test) (Fig. 16C,E), sdf1a morpholino alone, (32.8± 10.6%; n = 5 $p < 0.05$; t-test) (Fig. 16B,E), or wild type embryos (11.9 ± 4.6; n=8; $p < 0.05$; t-test) (Fig 16D,E). Taken together, our data are consistent with the idea that FBMNs must integrate multiple guidance cues to achieve persistent caudal directionality, including Cdh2-mediated soma-soma CIL dispersion and Sdf1/Cxcr4b-mediated chemotaxis.

Discussion

In this report, we establish a novel role for CIL in neuron migration as well as the need for multiple guidance cue integration. We aimed to determine the nature of physical interactions during collective FBMN migration. We utilized membrane bound GFP expression and high-resolution confocal imaging to visualize both FBMNs and axons in successive time points during early migration. At early time points (16-19hpf) neurons do not appear to require axon contact in order to migrate out of r4, as suggested by Wanner and Prince, 2013. Instead, neurons do appear to be making transient contact with each other during migration. This led us to investigate the nature of these transient interactions. We again used confocal microscopy, this time in live wild-type embryos to determine the outcome of these transient interactions. Upon further examination, we determined often when neurons contacted each other, migration briefly stopped and neurons migrated away from each other, characteristic of migration seen in other cell types exhibiting contact inhibition of locomotion (CIL).

We further investigated this by exploring the phenomena of CIL. CIL has been characterized in many cell types, including but not limited to cancer cells and wound healing *in vitro*, as well as neural crest cells *in vivo* (Roycroft and Mayor, 2015; Roycroft and Mayor, 2016). CIL has been described as a migration mechanism with four characteristic steps; 1) cells collide 2) protrusions collapse 3) protrusions reform in free space away from collision site 4) cells migrate away from each other (Roycroft and Mayor, 2016). Thus, we proposed if

FBMNs are engaging in CIL behaviour, we would be able to witness these characteristic events during live imaging. In fact, upon examining events when two cells contacted each other during caudal migration, cells halted and then proceeded to migrate in opposite directions 49% of the time. Additionally, using membrane-bound GFP expression in transgenic embryos, we also witnessed protrusions collapsing at the site of contact and re-forming in free space. Taken together, these results suggested to us that FBMNs do use CIL during caudal collective migration, which has never before been shown during neural development.

As previously mentioned, chemotaxis also plays a role in neuron migration. We aimed to confirm this and further characterize chemokine signalling specifically in FBMN migration. Upon examination of both *sdf1* morphants and *cxcr4b* mutants and quantification of neuron position at end time points, we observed a partial migration defect, where FBMNs are scattered between r4 and r6. This confirmed what previous studies (Cubedo et al., 2009; Sapède et al., 2005) reported as well as suggested to us that chemotaxis plays a role in FBMN migration.

We next suggested that integration of multiple signals is required for proper migration. Using live imaging of *sdf1* morphants, we witnessed in the absence of chemotaxis, there is greater CIL dispersion. This explains the spread out phenotype seen in cxcr4b mutants and sdf1 MO. It also suggests that SDF1 signaling overcomes CIL dispersion in some cells causing a higher percentage of non-CIL outcomes in wild-type embryos.

Previous data from Rebman et al., 2016 suggests that CIL is absolutely required for initiation of migration. In absence of neuron-neuron CIL interactions, FBMNs are unable to respond to Sdf1 and migrate caudally. This suggests that polarity and protrusions produced by CIL are required first. We propose that Sdf1 can then act on protrusions and stabilize them (and counteract re-direction caused by CIL) in the posterior direction. When using lower level of Cdh2 inactivation, the limited caudal movement is likely caused by Sdf1 signaling. This

limited caudal movement is abrogated when Sdf1 signaling is removed. This suggests that on their own, each signal is insufficient to promote full caudal migration, but neurons must integrate both cues to achieve persistent caudal migration. This suggests a model for collective migration of FBMNs in which CIL is required to induce protrusions for cells to become motile, which are then stabilized and attracted to r6 by Sdf1/Cxcr4b chemokine system (Fig. 17)

There is a one caveat to studying FBMN migration *in vivo*, much like any other *in vivo* study; the neurons are spatially constricted. Many CIL studies use cell culture models, where the environment is extremely simplified and normally one-dimensional. That is, cells contact and movement is in one plane(Lin et al., 2015) Cells migrating in a dish are not spatially constricted, thus collision outcome results are clearly witnessed. When two cells contact each other in cell culture, directional changes are easily witnessed. Unlike cell culture, FBMNs are migrating through the ventral neural tube, among neuroepithelial cells. At the same time, FBMNs are contacting each other and other cells, all in the presence of a live embryo and many conflicting cues. Thus, exploring CIL in this 3D environment, where cell outcomes result in anterior, posterior, dorsal or ventral movement, makes the presence of CIL seem less significant than that of cell culture models. When two migrating neurons contact each other, they attempt to migrate away from each other, but often there is another neuron in close proximity, which then causes another CIL event. Thus we propose CIL results in ‘traffic-jam’ like behaviour, where cells are constantly contacting each other and jostling around in the spatially restricted ventral neural tube. Regardless of the fact that CIL is less profound in FBMNs in vivo when compared to cell culture studies, it is in fact functioning to achieve proper FBMN directional migration.

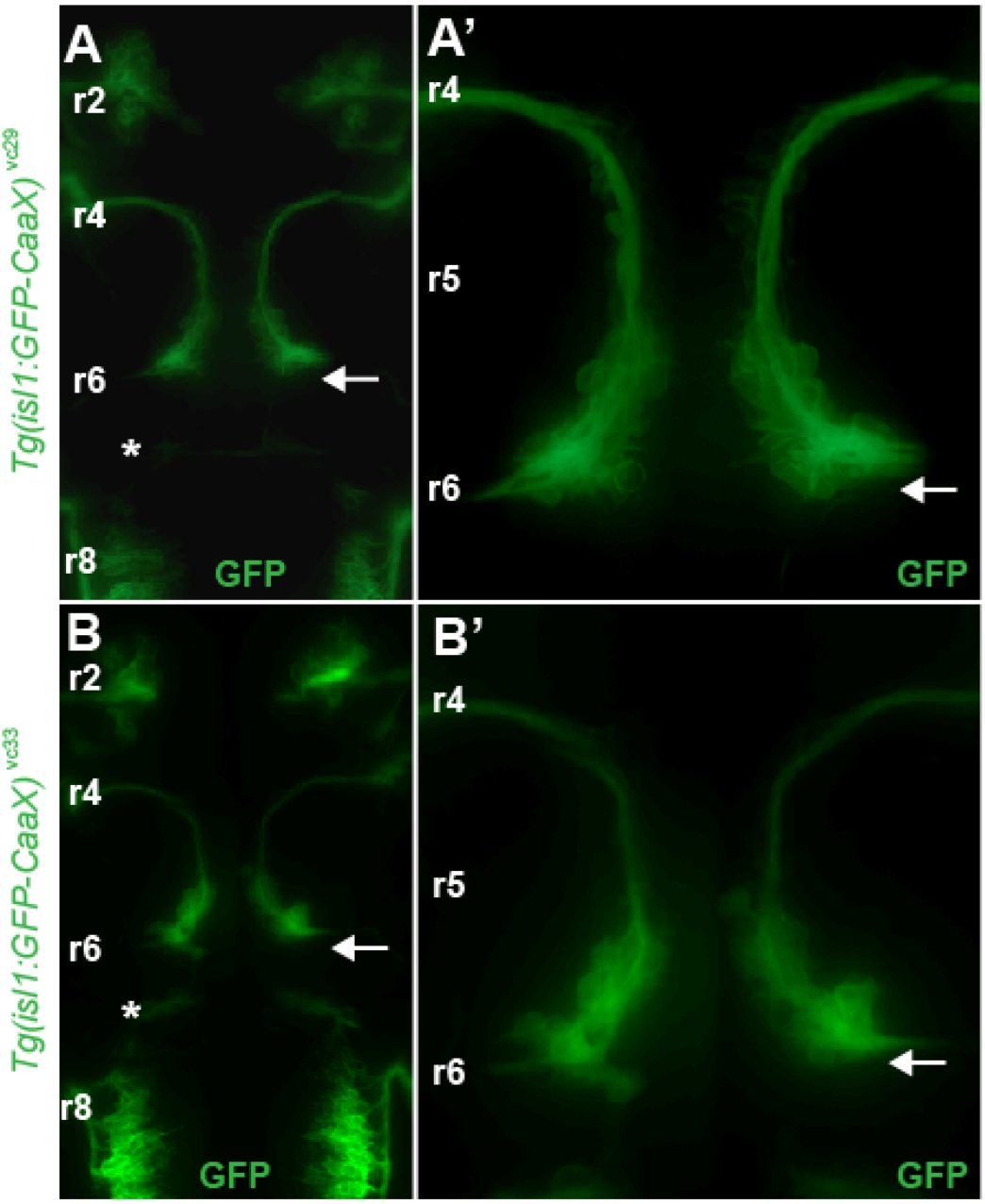

Fig. 10. **Generation of a stable transgenic fish expressing GFP-CaaX in cranial motor neurons.** (A-B') Live confocal imaging of embryos at 42 hpf from two different stable isl1:GFP-CaaX transgenic lines: *Tg(isl1:GFP-CaaX)*^{vc29} and *Tg(isl1:GFP-CaaX)*^{vc33}, respectively. Images represent transgene expression specifically in cranial motor neurons between r2 and r8 (A, B) note transgene expression in both neurons and axons of FBMNs (A', B'). Despite transgene expression, FBMNs migrate normally into r6 (white arrows) similar to wild type FBMNs. Similar to *Tg(isl1:GFP)* embryos, (discussed in introduction) white asterisk denotes PLL efferent neurons, which differ from r4-derived FBMN populations.

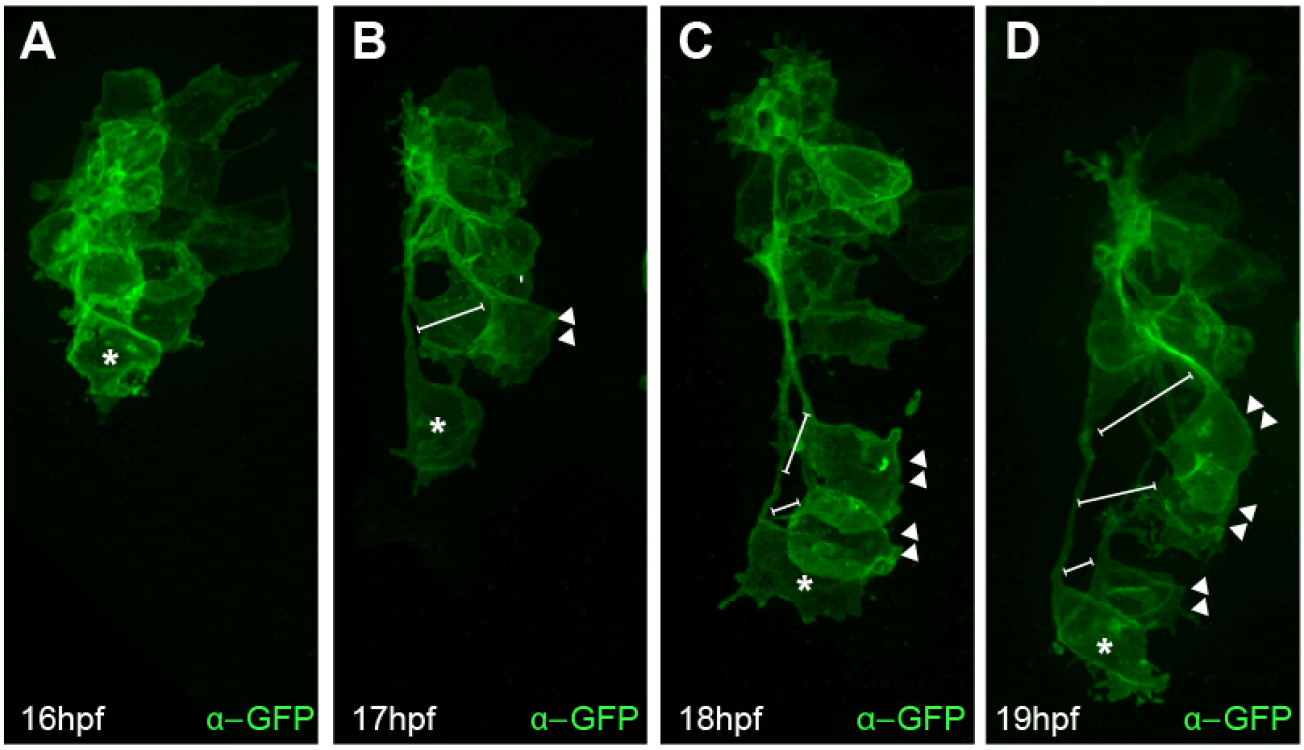

Fig. 11. Early born FBMNs make contact with other FBMNs while migrating. (A-D) Whole-mount immunocytochemistry showing dorsal views of early born FBMNs in *Tg(isl1:GFP-CaaX)* embryos as they are migrating out of r4. Embryos are labeled with α-GFP (green) antibody. Images represent close up of neurons when they are born at 16hpf and begin to migrate, followed by later migration stages of 17hpf, 18hpf and 19hpf. Neurons migrate independently of the leader neuron's axon (white asterisks). Note subsequent neurons migrating after leader (white arrowheads) make soma-soma contact and are not intimately associated with the leader neuron's axon (white brackets). (A) Shows neurons just being born and starting to migrate out of r4 where axon-soma verses soma-soma contact cannot yet be determined. (B-D) Show successive early migration where soma-soma contact is more prevalent than soma-axon contact.

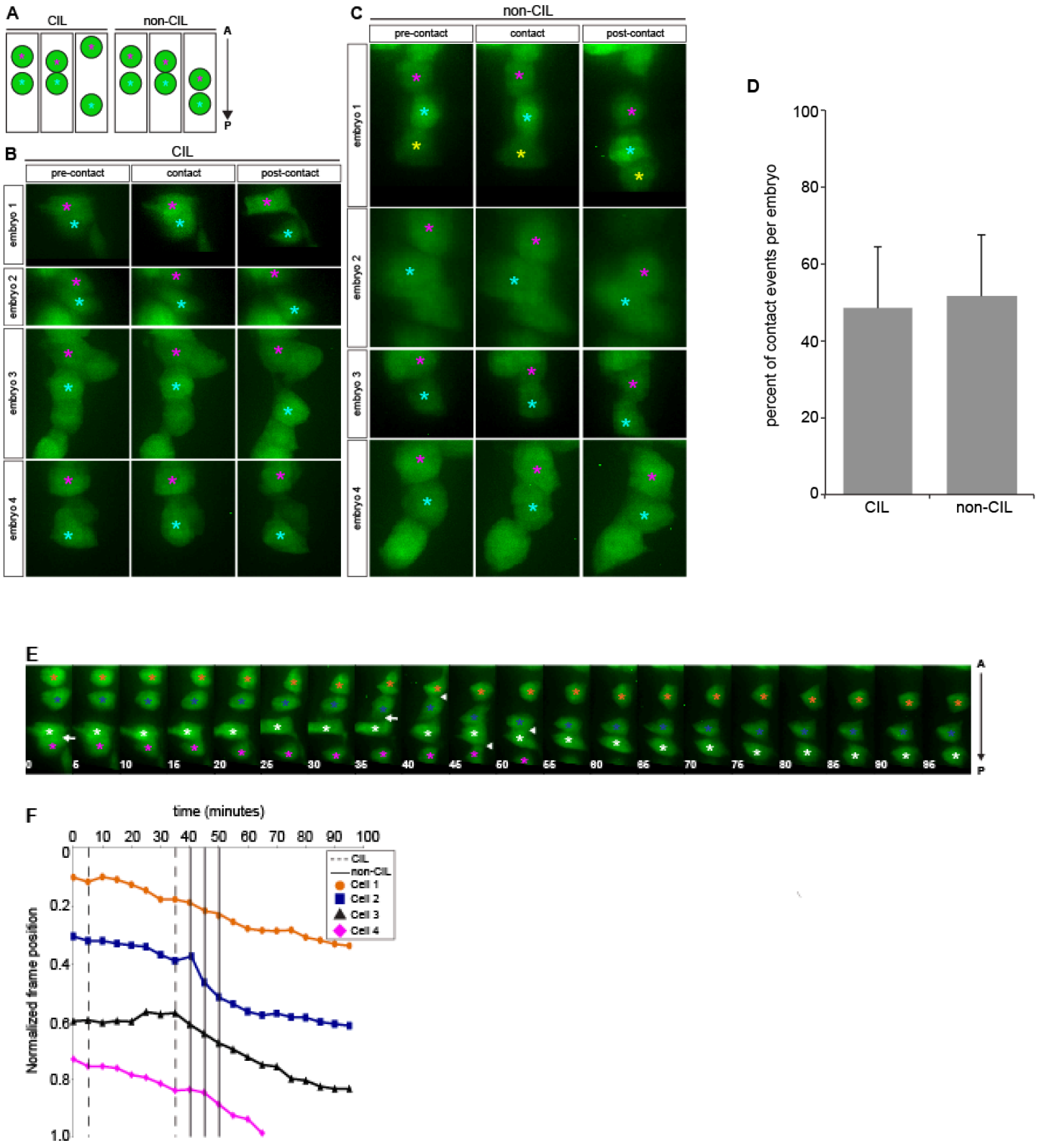

Fig. 12. FBMNs engage in CIL behavior during migration. (A) Schematic representation of two possible outcomes of cell-cell collisions, between migrating FBMNs pre-contact event, contact event and post-contact event. When two neurons contact each other (magenta and cyan asterisks), they briefly halt migration and then begin migrating again in the same direction (non-CIL outcome) or in opposite directions (CIL outcome). (B,C) Images show still frames

from confocal live imaging of various *Tg(isl1:GFP)* embryos from 16-22 hpf. Frames show neuron positioning before, during and after contact events and give representative examples of CIL (B) and non-CIL (C) outcomes in migrating FBMNs, respectively. (D) Quantification of cell-cell collision outcomes as a percentage of total cell-cell collisions per embryo. No statistically significant difference in CIL versus non-CIL outcomes (t-test). (E) Represents individual frames of confocal live imaging of *Tg(isl1:GFP)* embryo where migrating FBMNs are labeled in green migrating . Time course highlights cell-cell collision events with both CIL (white arrows) and non-CIL (white arrowheads) outcomes in four different migrating neurons over time (orange, blue, white and magenta asterisks). (F) Graph represents a quantification cell movement over time (min), with colored lines tracking the normalized frame position of each corresponding cell as denoted by colored asterisks. Black lines represent cell collisions where dashed lines represent CIL events and solid lines represent non-CIL events.

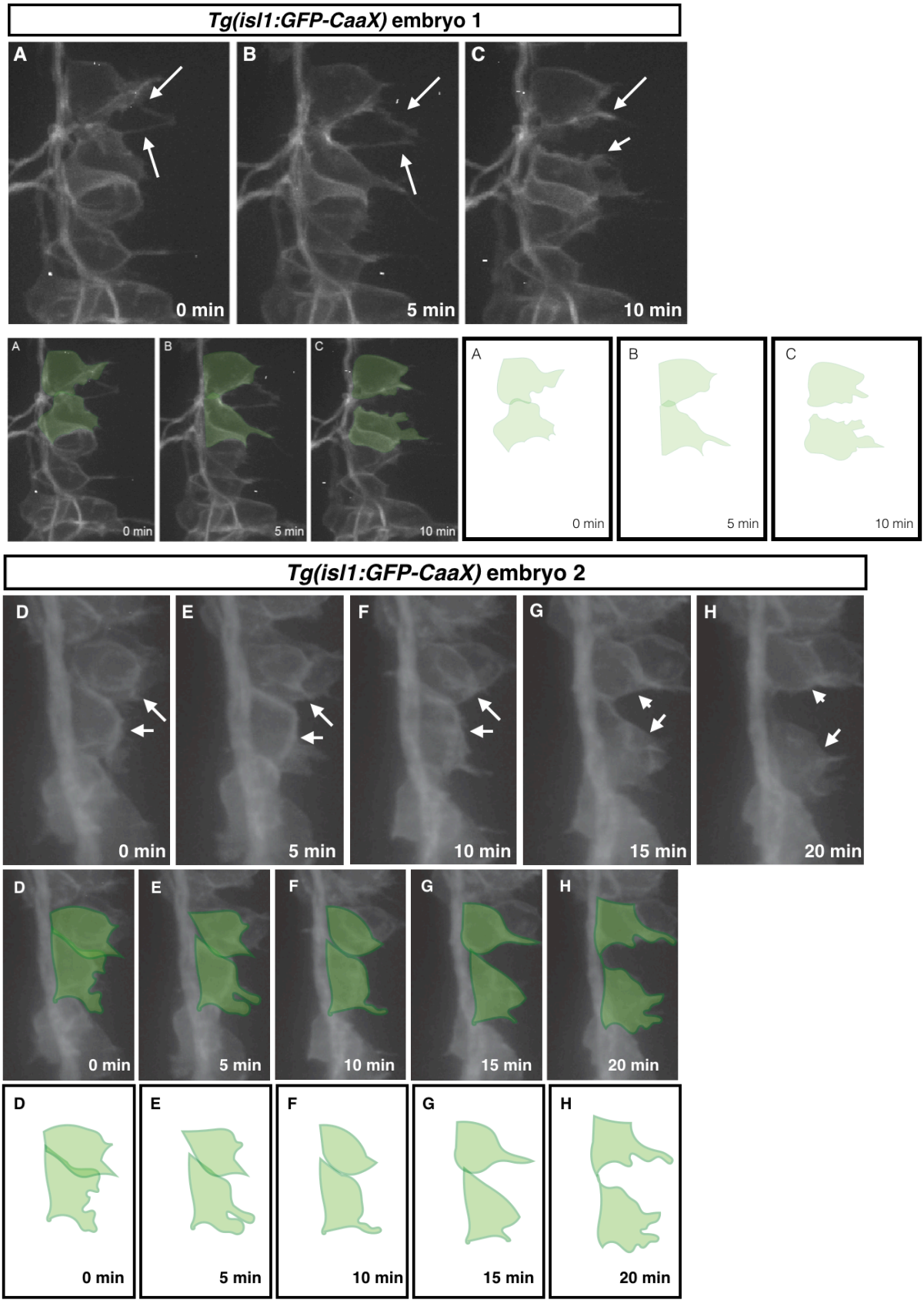

Fig. 13. **FBMNs exhibit protrusive changes after cell-cell contact.** (A-H) Two different examples of protrusive changes before, during and after collision events in isl1:GFP-CaaX

transgenic zebrafish.(A-C) Successive time frames during time-lapse live imaging of 22hpf embryo. Images show two migrating neurons contacting each other (A), collapsing protrusions near the site of contact (white arrows) (B) and finally moving away from each other and forming new protrusions in free space (C). (D-H) Successive time frames during time-lapse live imaging of 26hpf embryo. Images show two migrating neurons contacting each other (D), collapsing protrusions (white arrows) (E-G) and migrating in opposite directions (H).

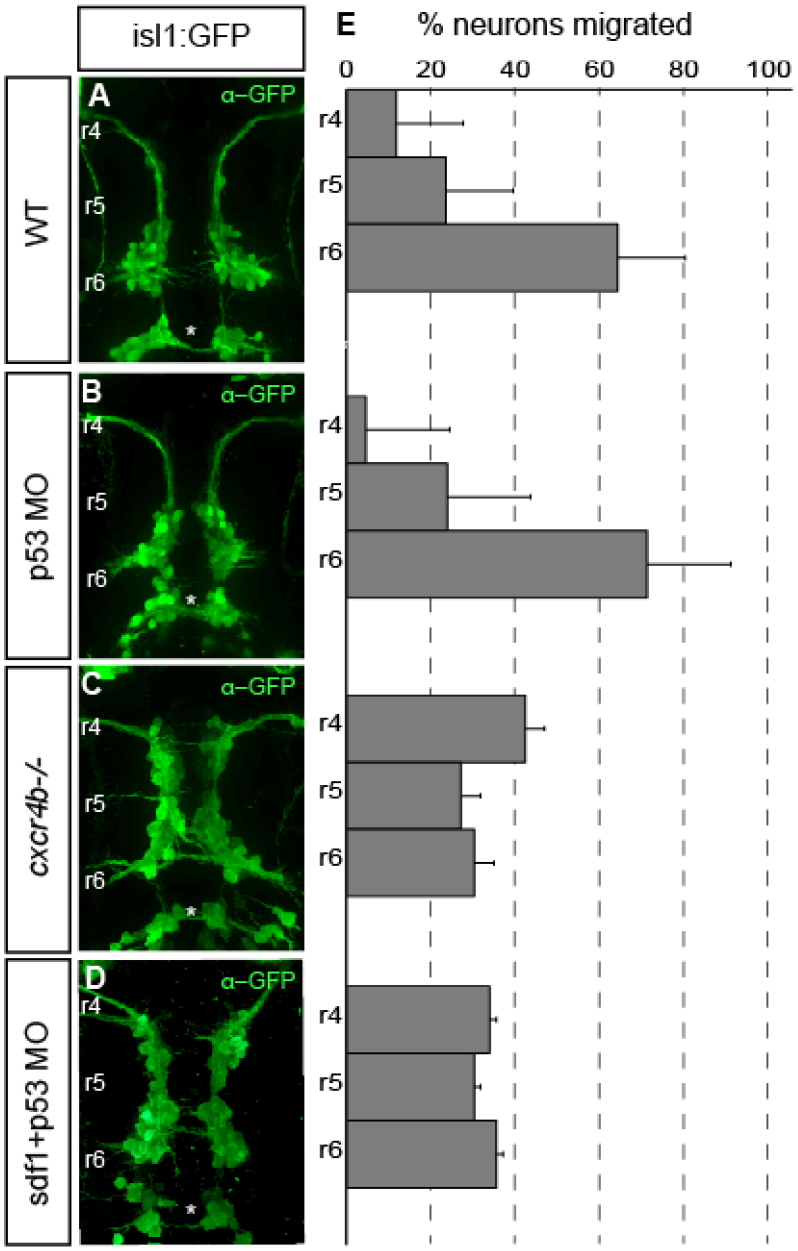

Fig. 14. Chemotaxis plays a role in sustained caudal migration of FBMNs. (A-D) Whole-mount immunocytochemistry showing dorsal views of *Tg(isl1:GFP)* transgenic embryos at 42 hpf embryos. Embryos are labeled with α-GFP (green). (A) Wild-type *Tg(isl1:GFP)* embryos with FBMNs fully migrated into r6. (B) Wild-type *Tg(isl1:GFP)* embryos injected with *p53* morpholino (control) with FBMNs fully migrated into r6, ruling out possibility of cell death contributing to migration. (C) Partial defect in caudal migration of FBMNs in cxcr4b -/- homozygous mutant *Tg(isl1:GFP)* embryos. (D) Partial defect in caudal migration of FBMNs in *Tg(isl1:GFP)* embryos injected with *sdf1a* morpholino combined with *p53* morpholino. (E) Histograms indicate the percent of FBMNs at 42 hpf that failed to migrate (r4), migrated partially (r5), or migrated fully (r6). Each histogram corresponds to the genetic condition in the image to its left and represents percent neurons migrated per embryo. (Mean values ± SD are shown; $p < 0.05$; t-test).

White asterisk denotes r6-derived PLL efferent neurons, which differ from r4-derived FBMN populations.

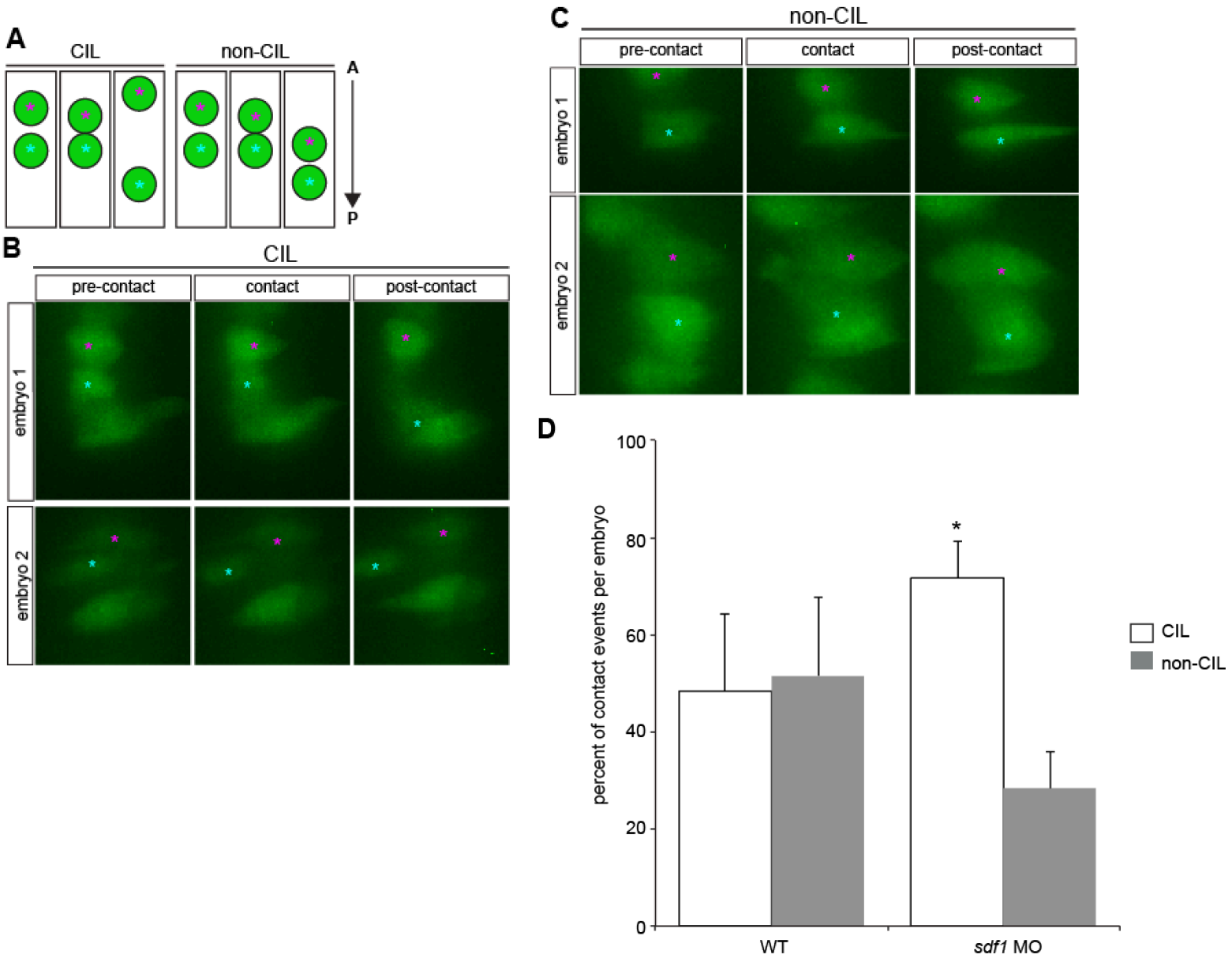

Fig. 15. FBMN cell-cell collision CIL outcomes increase when Sdf1 is knocked down. (A) Schematic representation of two possible outcomes of cell-cell collisions, between migrating FBMNs pre-contact event, contact event and post-contact event. When two neurons contact each other (magenta and cyan asterisks), they briefly halt migration and then begin migrating again in the same direction (non-CIL outcome) or in opposite directions (CIL outcome). (B,C) Images show still frames from confocal live imaging of various *Tg(isl1:GFP)* + Sdf1 MO embryos from 16-22 hpf. Frames show neuron positioning before, during and after contact events and give representative examples of CIL (B) and non-CIL (C) outcomes in migrating FBMNs, respectively. (D) Quantification of cell-cell collision outcomes as a percentage of total cell-cell collisions per embryo in wild-type embryos versus *sdf1* morphant embryos. Asterisk denotes statistical significance ($p<0.05$; t-test).

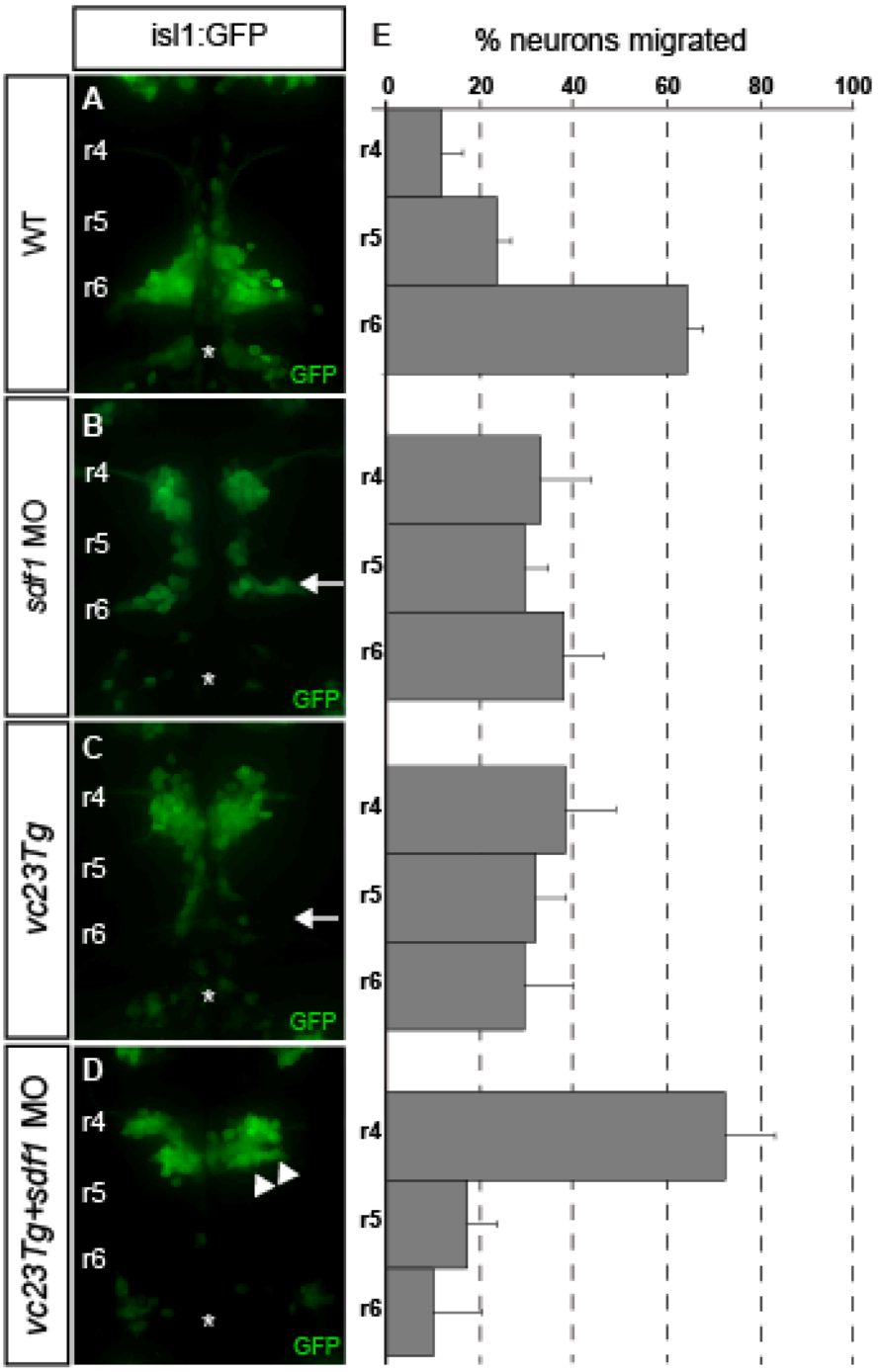

Fig. 16. FBMNs integrate multiple guidance cues to achieve proper caudal collective migration. (A-D) Confocal imaging showing dorsal views of live transgenic embryos at 42 hpf. Embryos are labeled with isl1:GFP transgene (green). (A) Wild-type *Tg(isl1:GFP)* embryos with FBMNs fully migrated into r6. (B) Partial defective caudal migration of FBMNs in *Tg(isl1:GFP) sdf1* morphant embryos. (C) Partial defective caudal migration of FBMNs in *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc23* embryos carrying one copy of the transgene (hemizygous). (D) Severe migration defect of FBMNs in hemizygous *Tg(isl1:GFP)/Tg(isl1:cdh2ΔEC-mCherry)vc23* embryos injected with *sdf1* morpholino. White arrows denote partial migration defect while white arrowheads denote severe migration defect. (J) Histograms indicate the percent of FBMNs at 42 hpf that failed to migrate (r4), migrated partially (r5), or migrated fully (r6). Each histogram corresponds to the genetic condition in the image to its left and represents the percent neurons migrated per embryo. (Mean values ± SD

are shown; $p < 0.05$;)White asterisk denotes r6-derived PLL efferent neurons, which differ from r4-derived FBMN populations.

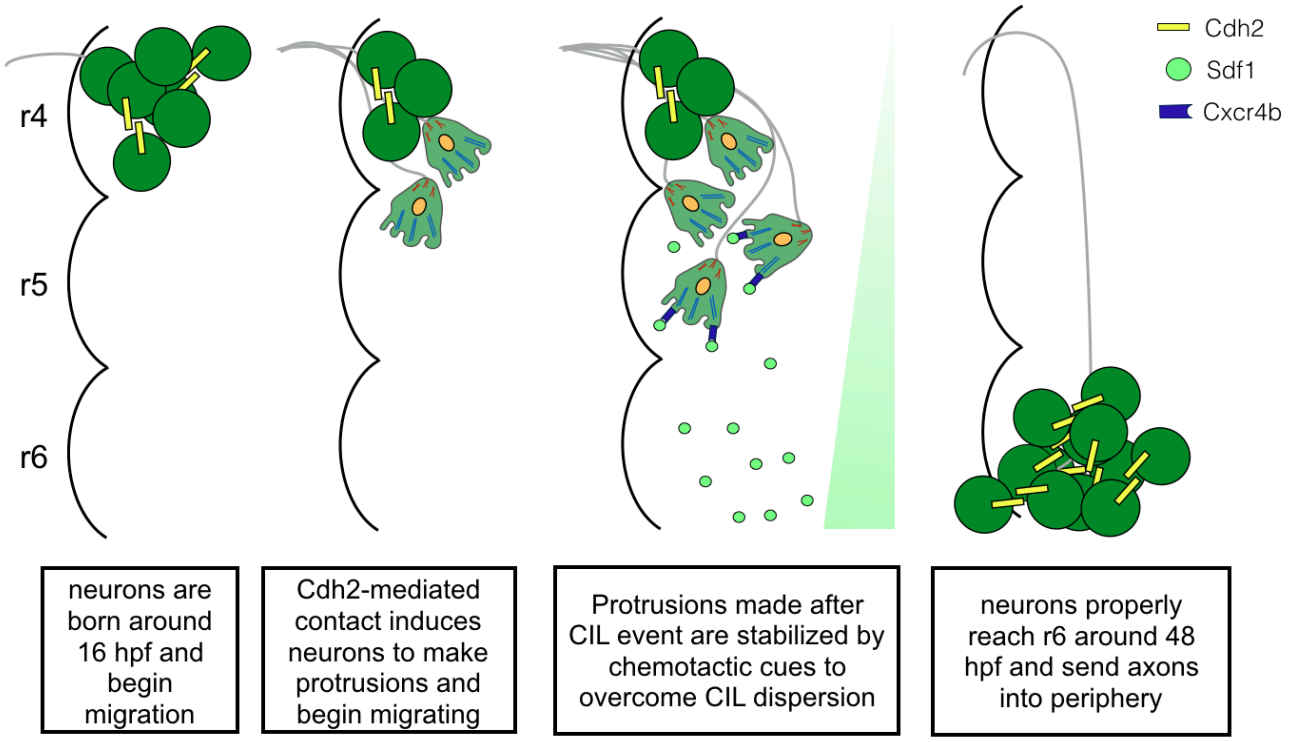

Fig. 17. Proposed model for collective migration of FBMNs. Schematic of FBMNs migrating out of r4 in a posterior direction. FBMNs contact each other via Cdh2-mediated signaling which induced protrusions to form. These protrusions are stabilized in the presence of Sdf1/Cxcr4b in order to achieve proper migration.

Future Directions

Neuronal migration is an essential process in the development and function of vertebrate organisms. The results presented in this thesis highlight the importance of a previously unexplored mode of FBMN migration, collective cell migration. Additionally, this thesis explored a novel role for CIL in neural development. Although it has made a critical impact for the field of neural development and cell migration, there are more studies that should be conducted to further confirm the conclusions drawn.

While dominant-negative approaches are widely used and helped us to create a tissue-specific knockdown of Cadherin-2, it would be of interest to create a tissue-specific CRISPR mutant for Cdh-2 in cranial motor neurons. As our findings supported, the amount of transgene expressed was correlated directly with the migration defect we witnessed. Thus, the phenotype was variable depending upon how much of the transgene was expressed. This could be problematic when looking to explore early time points of migration, as once the transgene is expressed at a high enough level to visualize, neurons have already began to migrate. Using a tissue-specific CRISPR mutant would help to avoid the problems associated with dominant negative transgenesis.

Additionally, it is critical to explore how Cdh2 is controlling collective migration. It is likely similar to other systems (neural crest) where Cdh2 cell contact on neighboring cells leads to an intracellular signaling cascade ultimately acting on Rac1/RhoA GTPase. In neural crest cells, Cdh2 contact activates RhoA to collapse protrusions at the site of contact (Theveneau and Mayor, 2010). In order to explore RhoA in FBMNs, I would propose to first create a construct that drives GFP expression in the presence of RhoA activity. I would use live imaging of isl1:memRFP embryos to visualize cell-cell contact events, which would highlight membrane protrusions in red. I would expect that collision events resulting in CIL would show rapid RhoA activity (green) at sites of cell-cell contact, indicating that protrusion collapse during CIL is due

to RhoA activity. I would then conduct a similar experiment in sdf1 MO embryos to determine the changes in RhoA activity between wild-type and morphant neurons. I would expect morphant embryos to exhibit increased levels of RhoA activity when compared to wild-type FBMNs, as Sdf1 has been associated with protrusion stability in other cell types (Theveneau et al., 2010). This would perhaps lend a further explanation of the partial migration defect we see in *sdf1* morphant embryos.

Furthermore, I would like to characterize specific protrusion dynamics in migrating FBMNs. While spinning disc confocal microscopy allows live imaging of transgenic zebrafish, it is limited by the signal of a fluorescent transgene at early migration time points. During early migration, neurons have just been born and begin to migrate before building up sufficient transgene levels to be excited by lasers on spinning disc confocal microscopes, thus exposure rates must be very high to witness transgene. This limits the amount of pictures that can be taken over time, in order to not photo bleach the sample and kill the embryo. In past live imaging conducted for this thesis, imaging was limited to one frame per five minutes. Thus, any changes occurring between those five-minute intervals were missed. I would like to explore using light sheet microscopy to live image transgenic zebrafish in order to take high-resolution images at high frame rates to explore protrusive dynamics further. It would be interesting to measure how protrusions change over time, the direction they project in, and how quickly they assemble and dissemble.

Although there is much more to be done, the studies involved in this thesis will greatly impact the field of neural development. It has shown evidence of Cdh2-mediated collective migration in FBMNs. It has also established a novel role for CIL in neuron migration. I look forward to seeing other students further study these phenomena and taking this project forward in the future.

References

Abercrombie, M. (1979). Contact Inhibiton and malignancy. *Nature* **281**, 259–262.

Aberle, H., Butz, S., Stappert, J., Weissig, H., Kemler, R. and Hoschuetzky, H. (1994). Assembly of the cadherin-catenin complex in vitro with recombinant proteins. *J. Cell Sci.* **107 (Pt 12)**, 3655–63.

Affolter, M. and Weijer, C. J. (2005). Signaling to cytoskeletal dynamics during chemotaxis. *Dev. Cell* **9**, 19–34.

Arboleda-Estudillo, Y., Krieg, M., Stühmer, J., Licata, N. a, Muller, D. J. and Heisenberg, C.-P. (2010). Movement directionality in collective migration of germ layer progenitors. *Curr. Biol.* **20**, 161–9.

Arikkath, J., Cox, D. N. and Mason, G. (2012). Molecular mechanisms of dendrite morphogenesis. **6**, 1–14.

Astin, J. W., Batson, J., Kadir, S., Charlet, J., Persad, R. A., Gillatt, D., Oxley, J. D. and Nobes, C. D. (2010). Competition amongst Eph receptors regulates contact inhibition of locomotion and invasiveness in prostate cancer cells. *Nat Cell Biol* **12**, 1194–1204.

Beattie, R. and Hippenmeyer, S. (2017). Mechanisms of radial glia progenitor cell lineage progression. *FEBS Lett.* **591**, 3993–4008.

Benes, F. M. and Berretta, S. (2001). GABAergic Interneurons: Implications for Understanding Schizophrenia and Bipolar Disorder. *Neuropsychopharmacology* **25**, 1.

Bingham, S., Higashijima, S., Okamoto, H. and Chandrasekhar, A. (2002). The Zebrafish trilobite gene is essential for tangential migration of branchiomotor neurons. *Dev. Biol.* **242**, 149–60.

Brusés, J. L. (2011). N-cadherin regulates primary motor axon growth and branching during zebrafish embryonic development. *J. Comp. Neurol.* **519**, 1797–815.

Carmona-Fontaine, C., Matthews, H. K., Kuriyama, S., Moreno, M., Dunn, G. A., Parsons,

M., Stern, C. D. and Mayor, R. (2008). Contact inhibition of locomotion in vivo controls neural crest directional migration. *Nature* **456**, 957–61.

Carreira-Barbosa, F., Concha, M., Takeuchi, M., Ueno, N., Wilson, S. W. and Tada, M. (2003). Prickle 1 regulates cell movements during gastrulation and neuronal migration in zebrafish. *Development* **130**, 4037–4046.

Carvajal-Gonzalez, J. M. and Mlodzik, M. (2014). Mechanisms of planar cell polarity establishment in Drosophila. *F1000Prime Rep.* **6**,.

Chandrasekhar, A. (2004). Turning heads: development of vertebrate branchiomotor neurons. *Dev. Dyn.* **229**, 143–61.

Charest, P. G. and Firtel, R. A. (2006). Feedback signaling controls leading-edge formation during chemotaxis. *Curr. Opin. Genet. Dev.* **16**, 339–347.

Cubedo, N., Cerdan, E., Sapede, D. and Rossel, M. (2009). CXCR4 and CXCR7 cooperate during tangential migration of facial motoneurons. *Mol. Cell. Neurosci.* **40**, 474–484.

Das, G., Jenny, A., Klien, T. J., Eaton, S. and Mlodzik, M. (2004). Diego interacts with Prickle and Strabismus/Van Gogh to localize planar cell polarity complexes. *Development* **131**, 4467–4476.

Davey, C. F. and Moens, C. B. (2017). Planar cell polarity in moving cells: think globally, act locally. *Development* **144**, 187–200.

Davey, C. F., Mathewson, A. W. and Moens, C. B. (2016). PCP Signaling between Migrating Neurons and their Planar-Polarized Neuroepithelial Environment Controls Filopodial Dynamics and Directional Migration. *PLoS Genet.* **12**, 1–30.

David, N. B., Sapede, D., Saint-Etienne, L., Thisse, C., Thisse, B., Dambly-Chaudiere, C., Rosa, F. M. and Ghysen, A. (2002). Molecular basis of cell migration in the fish lateral line: Role of the chemokine receptor CXCR4 and of its ligand, SDF1. *Proc. Natl. Acad. Sci.* **99**, 16297–16302.

Davis, J. R., Huang, C.-Y., Zanet, J., Harrison, S., Rosten, E., Cox, S., Soong, D. Y., Dunn, G. A. and Stramer, B. M. (2012). Emergence of embryonic pattern through contact inhibition of locomotion. *Development* **139**, 4555–4560.

Deisboeck, T. S. and Couzin, I. D. (2009). Collective behavior in cancer cell populations. *BioEssays* **31**, 190–197.

Denaxa, M., Chan, C. H., Schachner, M., Parnavelas, J. G. and Karagogeos, D. (2001). The adhesion molecule TAG-1 mediates the migration of cortical interneurons from the ganglionic eminence along the corticofugal fiber system. *Development* **128**, 4635–44.

Derycke, L. D. M., Bracke, M. E. and Bracke, M. (2004). N-cadherin in the spotlight of cell-cell adhesion, differentiation, embryogenesis, invasion and signalling Migration and invasion. *Int. J. Dev. Biol* **48**, 463–476.

Dumortier, J. G., Martin, S., Meyer, D., Rosa, F. M. and David, N. B. (2012a). Collective mesendoderm migration relies on an intrinsic directionality signal transmitted through cell contacts. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 16945–16950.

Dumortier, J. G., Martin, S., Meyer, D., Rosa, F. M. and David, N. B. (2012b). Collective mesendoderm migration relies on an intrinsic directionality signal transmitted through cell contacts. *Proc. Natl. Acad. Sci. U. S. A.* **109**, 16945–50.

Evsyukova, I., Plestant, C. and Anton, E. S. (2013). Integrative Mechanisms of Oriented Neuronal Migration in the Developing Brain. *Annu. Rev. Cell Dev. Biol.* **29**,.

Ezin, A. M., Fraser, S. E. and Bronner-Fraser, M. (2009). Fate map and morphogenesis of presumptive neural crest and dorsal neural tube. *Dev. Biol.* **330**, 221–236.

Fetcho, J. R., Higashijima, S. and McLean, D. L. (2008). Zebrafish and motor control over the last decade. *Brain Res. Rev.* **57**, 86–93.

Fu, C., Cawthon, B., Clinkscales, W., Bruce, A., Winzenburger, P. and Ess, K. C. (2011). GABAergic Interneuron Development and Function Is Modulated by the Tsc1 Gene.

Cereb. Cortex **22**, 2111–2119.

Garel, S., Garcia-Dominguez, M. and Charnay, P. (2000). Control of the migratory pathway of facial branchiomotor neurones. *Development* **127**, 5297–307.

Glasco, D. M., Sittaramane, V., Bryant, W., Fritzsch, B., Sawant, A., Paudyal, A., Stewart, M., Andre, P., Cadete Vilhais-Neto, G., Yang, Y., et al. (2012). The mouse Wnt/PCP protein Vangl2 is necessary for migration of facial branchiomotor neurons, and functions independently of Dishevelled. *Dev. Biol.* **369**, 211–22.

Grant, P. K. and Moens, C. B. (2010). The neuroepithelial basement membrane serves as a boundary and a substrate for neuron migration in the zebrafish hindbrain. *Neural Dev.* **5**, 1–17.

Guan, K. L. and Rao, Y. (2003). Signalling mechanisms mediating neuronal responses to guidance cues. *Nat. Rev. Neurosci.* **4**, 941–956.

Guthrie, S. (1996). Patterning the hindbrain. *Curr. Opin. Neurobiol.* **6**, 41–48.

Haastert, P. J. M. Van and Devreotes, P. N. (2004). CHEMOTAXIS: SIGNALLING THE WAY FORWARD. **5**,.

Heisenberg, C.-P. and Tada, M. (2002). Zebrafish gastrulation movements: bridging cell and developmental biology. *Semin. Cell Dev. Biol.* **13**, 471–479.

Higashijima, S., Hotta, Y. and Okamoto, H. (2000). Visualization of cranial motor neurons in live transgenic zebrafish expressing green fluorescent protein under the control of the islet-1 promoter/enhancer. *J. Neurosci.* **20**, 206–18.

Hong, E. and Brewster, R. (2006). N-cadherin is required for the polarized cell behaviors that drive neurulation in the zebrafish. *Development* **133**, 3895–905.

Hunter, M. V., Lee, D. M., Harris, T. J. C. and Fernandez-Gonzalez, R. (2015). Polarized E-cadherin endocytosis directs actomyosin remodeling during embryonic wound repair. *J. Cell Biol.* **210**, 801–816.

Hutchins, J. B. and Barger, S. W. (1998). Why Neurons Die : Cell Death in the Nervous System.

Ireton, R. C., Davis, M. A., Van Hengel, J., Mariner, D. J., Barnes, K., Thoreson, M. A., Anastasiadis, P. Z., Matrisian, L., Bundy, L. M., Sealy, L., et al. (2002). A novel role for p120 catenin in E-cadherin function. *J. Cell Biol.* **159**, 465–476.

Jessen, J. R., Topczewski, J., Bingham, S., Sepich, D. S., Marlow, F., Chandrasekhar, A. and Solnica-Krezel, L. (2002). Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. **4**, 610–615.

Jontes, J. D., Emond, M. R. and Smith, S. J. (2004). In vivo trafficking and targeting of N-cadherin to nascent presynaptic terminals. *J. Neurosci.* **24**, 9027–34.

Kai, M., Heisenberg, C.-P. and Tada, M. (2008). Sphingosine-1-phosphate receptors regulate individual cell behaviours underlying the directed migration of prechordal plate progenitor cells during zebrafish gastrulation. *Development* **135**, 3043–3051.

Kawakami, K. (2007). Tol2: A versatile gene transfer vector in vertebrates. *Genome Biol.* **8**, 1–10.

Kawauchi, T., Sekine, K., Shikanai, M., Chihama, K., Tomita, K., Kubo, K., Nakajima, K., Nabeshima, Y. and Hoshino, M. (2010). Rab GTPases-Dependent Endocytic Pathways Regulate Neuronal Migration and Maturation through N-Cadherin Trafficking. *Neuron* **67**, 588–602.

Kennedy, T. E. (2000). Cellular mechanisms of netrin function: Long-range and short-range actions. *Biochem Cell Biol* **78**, 569–75.

Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310.

Kishimoto, Y., Lee, K. H., Zon, L., Hammerschmidt, M., Schulte-Merker, S., Peters, K., Grunwald, D. J., Stainier, D. Y. R., Jiang, Y.-J. and Heisenberg, C.-P. (1997). The

molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. *Development* **124**, 4457–66.

Knaut, H., Werz, C., Geisler, R., The Tübingen 2000 Screen Consortium and Nüsslein-Volhard, C. (2002). A zebrafish homologue of the chemokine receptor CXCR4 is a germ-cell guidance receptor. *Nature* **421**, 279.

Lele, Z., Folchert, A., Concha, M., Rauch, G.-J., Geisler, R., Rosa, F., Wilson, S. W., Hammerschmidt, M. and Bally-Cuif, L. (2002). Parachute/N-Cadherin Is Required for Morphogenesis and Maintained Integrity of the Zebrafish Neural Tube. *Development* **129**, 3281–94.

Lewellis, S. W., Nagelberg, D., Subedi, A., Staton, A., LeBlanc, M., Giraldez, A. and Knaut, H. (2013). Precise SDF1-mediated cell guidance is achieved through ligand clearance and microRNA-mediated decay. *J. Cell Biol.* **200**, 337–355.

Lin, B., Yin, T., Wu, Y. I., Inoue, T. and Levchenko, A. (2015). Interplay between chemotaxis and contact inhibition of locomotion determines exploratory cell migration. *Nat. Commun.* **6**, 1–14.

Luccardini, C., Hennekinne, L., Viou, L., Yanagida, M., Murakami, F., Kessaris, N., Ma, X., Adelstein, R. S., Mège, R.-M. and Métin, C. (2013). N-cadherin sustains motility and polarity of future cortical interneurons during tangential migration. *J. Neurosci.* **33**, 18149–60.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science (80-.).* **274**, 1109–1115.

Maness, P. F. and Schachner, M. (2007). Neural recognition molecules of the immunoglobulin superfamily: Signaling transducers of axon guidance and neuronal migration. *Nat. Neurosci.* **10**, 19–26.

Mapp, O. M., Wanner, S. J., Rohrschneider, M. R. and Prince, V. E. (2010). Prickle1b

mediates interpretation of migratory cues during zebrafish facial branchiomotor neuron migration. *Dev. Dyn.* **239**, 1596–608.

Mapp, O. M., Walsh, G. S., Moens, C. B., Tada, M. and Prince, V. E. (2011). Zebrafish Prickle1b mediates facial branchiomotor neuron migration via a farnesylation-dependent nuclear activity. *Development* **138**, 2121–32.

Marín, O., Valiente, M., Ge, X. and Tsai, L.-H. (2010). Guiding neuronal cell migrations. *Cold Spring Harb. Perspect. Biol.* **2**, a001834.

Martinez-Garay, I., Gil-Sanz, C., Franco, S. J., Espinosa, A., Molnár, Z. and Mueller, U. (2016). Cadherin 2/4 signaling via PTP1B and catenins is crucial for nucleokinesis during radial neuronal migration in the neocortex. *Development* **143**, 2121–2134.

Mayor, R. and Carmona-Fontaine, C. (2010). Keeping in touch with contact inhibition of locomotion. *Trends Cell Biol.* **20**, 319–328.

Mayor, R. and Etienne-Manneville, S. (2016). The front and rear of collective cell migration. *Nat. Rev. Mol. Cell Biol.* **17**, 97–109.

McClintock, J. M., Kheirbek, M. a and Prince, V. E. (2002). Knockdown of duplicated zebrafish hoxb1 genes reveals distinct roles in hindbrain patterning and a novel mechanism of duplicate gene retention. *Development* **129**, 2339–2354.

Meglio, T. Di and Rijli, F. M. (2013). *Transcriptional Regulation of Tangential Neuronal Migration in the Vertebrate Hindbrain*. Elsevier Inc.

Montell, D. J. (2003). Border-cell migration: The race is on. *Nat. Rev. Mol. Cell Biol.* **4**, 13–24.

Nadarajah, B., Brunstrom, J. E., Grutzendler, J., Wong, R. O. L. and Pearlman, A. L. (2001). Two modes of radial migration in early development of the cerebral cortex. *Nat. Neurosci.* **4**, 143–150.

Naiche, L. A., Harrelson, Z., Kelly, R. G. and Papaioannou, V. E. (2005). T-Box Genes in Vertebrate Development.

Nieman, M. T., Kim, J., Johnson, K. R. and Wheelock, M. J. (1999). Mechanism of extracellular domain-deleted dominant negative cadherins. **1632**, 1621–1632.

Niessen, C. M., Leckband, D. and Yap, A. S. (2011). Tissue Organization by Cadherin Adhesion Molecules: Dynamic Molecular and Cellular Mechanisms of Morphogenetic Regulation. *Physiol. Rev.* **91**, 691–731.

Pan, X., Sittaramane, V., Gurung, S. and Chandrasekhar, A. (2014). Structural and temporal requirements of Wnt/PCP protein Vangl2 function for convergence and extension movements and facial branchiomotor neuron migration in zebrafish. *Mech. Dev.* **131**, 1–14.

Pata, I., Studer, M., van Doorninck, J. H., Briscoe, J., Kuuse, S., Engel, J. D., Grosveld, F. and Karis, A. (1999). The transcription factor GATA3 is a downstream effector of Hoxb1 specification in rhombomere 4. *Development* **126**, 5523–31.

Pocock, R., Mione, M., Hussain, S., Maxwell, S., Pontecorvi, M., Aslam, S., Gerrelli, D., Sowden, J. C. and Woollard, A. (2008). Neuronal function of Tbx20 conserved from nematodes to vertebrates. *Dev. Biol.* **317**, 671–685.

Prince, V. E., Moens, C. B., Kimmel, C. B. and Ho, R. K. (1998). Zebrafish hox genes: expression in the hindbrain region of wild-type and mutants of the segmentation gene, valentino. *Development* **125**, 393–406.

Qu, Y., Glasco, D. M., Zhou, L., Sawant, A., Ravni, A., Fritzsch, B., Damrau, C., Murdoch, J. N., Evans, S., Pfaff, S. L., et al. (2010). Atypical cadherins Celsr1-3 differentially regulate migration of facial branchiomotor neurons in mice. *J. Neurosci.* **30**, 9392–401.

Radice, G. L., Rayburn, H., Matsunami, H., Knudsen, K. A., Takeichi, M. and Hynes, R. O. (1997a). Developmental defects in mouse embryos lacking N-cadherin. *Dev. Biol.* **181**, 64–78.

Radice, G. L., Rayburn, H., Matsunami, H., Knudsen, K. A., Takeichi, M. and Hynes, R. O.

(1997b). Developmental defects in mouse embryos lacking N-cadherin. *Dev. Biol.* **181**, 64–78.

Rebman, J. K., Kirchoff, K. E. and Walsh, G. S. (2016). Cadherin-2 Is Required Cell Autonomously for Collective Migration of Facial Branchiomotor Neurons. 1–18.

Richardson, J., Gauert, A., Briones Montecinos, L., Fanlo, L., Alhashem, Z. M., Assar, R., Marti, E., Kabla, A., Härtel, S. and Linker, C. (2016). Leader Cells Define Directionality of Trunk, but Not Cranial, Neural Crest Cell Migration. *Cell Rep.* **15**, 2076–2088.

Ridley, A. J. (2003). Cell Migration: Integrating Signals from Front to Back. *Science (80-.).* **302**, 1704–1709.

Rieger, S., Senghaas, N., Walch, A. and Köster, R. W. (2009). Cadherin-2 controls directional chain migration of cerebellar granule neurons. *PLoS Biol.* **7**,.

Rohrschneider, M. R., Elsen, G. E. and Prince, V. E. (2007). Zebrafish Hoxb1a regulates multiple downstream genes including prickle1b. *Dev. Biol.* **309**, 358–72.

Rørth, P. (2009). Collective Cell Migration. *Annu. Rev. Cell Dev. Biol* **25**, 407–429.

Rørth, P. (2011). Whence directionality: guidance mechanisms in solitary and collective cell migration. *Dev. Cell* **20**, 9–18.

Ross, M. E. and Walsh, C. A. (2001). Human brain malformations and their lessons for neuronal migration. **24**, 1041–1070.

Roycroft, A. and Mayor, R. (2015). Forcing contact inhibition of locomotion. *Trends Cell Biol.* **25**, 1–3.

Roycroft, A. and Mayor, R. (2016). Molecular basis of contact inhibition of locomotion. *Cell. Mol. Life Sci.* **73**, 1119–1130.

Rubenstein, J. L. R. and Marín, O. (2003). Cell Migration in the Forebrain. *Annu Rev Neurosci* **46**, 441–483.

Samad, O. A., Geisen, M. J., Caronia, G., Varlet, I., Zappavigna, V., Ericson, J., Goridis,

C. and Rijli, F. M. (2004). Integration of anteroposterior and dorsoventral regulation of Phox2b transcription in cranial motoneuron progenitors by homeodomain proteins. *Development* **131**, 4071–4083.

Sapède, D., Rossel, M., Dambly-Chaudière, C. and Ghysen, A. (2005). Role of SDF1 chemokine in the development of lateral line efferent and facial motor neurons. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 1714–8.

Scarpa, E. and Mayor, R. (2016). Collective cell migration in development. *J. Cell Biol.* **212**, 143–155.

Schmitt, A. M., Shi, J., Wolf, A. M., Lu, C. C., King, L. A. and Zou, Y. (2006). Wnt-Ryk signalling mediates medial-lateral retinotectal topographic mapping. *Nature* **439**, 31–37.

Shikanai, M., Nakajima, K. and Kawauchi, T. (2011). N-cadherin regulates radial glial fiber-dependent migration of cortical locomoting neurons. *Commun. Integr. Biol.* **4**, 326–30.

Sittaramane, V., Sawant, A., Wolman, M. A., Maves, L., Halloran, M. C. and Chandrasekhar, A. (2009). The cell adhesion molecule Tag1 , transmembrane protein Stbm / Vangl2 , and Laminin α 1 exhibit genetic interactions during migration of facial branchiomotor neurons in zebra fi sh. *Dev. Biol.* **325**, 363–373.

Song, M.-R. (2007). Moving cell bodies: understanding the migratory mechanism of facial motor neurons. *Arch. Pharm. Res.* **30**, 1273–82.

Song, M.-R., Shirasaki, R., Cai, C.-L., Ruiz, E. C., Evans, S. M., Lee, S.-K. and Pfaff, S. L. (2006). T-Box transcription factor Tbx20 regulates a genetic program for cranial motor neuron cell body migration. *Development* **133**, 4945–55.

Stemmler, M. P. (2008). Cadherins in development and cancer. *Mol. Biosyst.* **4**, 835–850.

Stockinger, P., Maître, J.-L. and Heisenberg, C.-P. (2011). Defective neuroepithelial cell cohesion affects tangential branchiomotor neuron migration in the zebrafish neural tube. *Development* **138**, 4673–83.

Stramer, B. and Mayor, R. (2016). Mechanisms and in vivo functions of contact inhibition of locomotion. *Nat. Publ. Gr.*

Stramer, B., Moreira, S., Millard, T., Evans, I., Huang, C. Y., Sabet, O., Milner, M., Dunn, G., Martin, P. and Wood, W. (2010). Clasp-mediated microtubule bundling regulates persistent motility and contact repulsion in Drosophila macrophages in vivo. *J. Cell Biol.* **189**, 681–689.

Suzuki, S. C. and Takeichi, M. (2008). Cadherins in neuronal morphogenesis and function. *Dev. Growth Differ.* **50**,.

Tada, M. and Kai, M. (2012). *Planar cell polarity in coordinated and directed movements.* 1st ed. Elsevier Inc.

Takeichi, M. (1995). Morphogenetic roles of classic cadherins. *Curr. Opin. Cell Biol.* **7**, 619–627.

Taniguchi, H., Kawauchi, D., Nishida, K. and Murakami, F. (2006). Classic cadherins regulate tangential migration of precerebellar neurons in the caudal hindbrain. *Development* **133**, 1923–31.

Tessier-Lavigne, Marc; Goodman, C. S. (1996). The Molecular Biology of Axon Guidance. *Science (80-.).* **274**, 1123–1133.

Theveneau, E. and Mayor, R. (2010). Integrating chemotaxis and contact-inhibition during collective cell migration: Small GTPases at work. *Small GTPases* **1**, 113–117.

Theveneau, E. and Mayor, R. (2012a). Neural crest migration: interplay between chemorepellents, chemoattractants, contact inhibition, epithelial-mesenchymal transition, and collective cell migration. *Wiley Interdiscip. Rev. Dev. Biol.* **1**, 435–45.

Theveneau, E. and Mayor, R. (2012b). Cadherins in collective cell migration of mesenchymal cells. *Curr. Opin. Cell Biol.* **24**, 677–84.

Theveneau, E. and Mayor, R. (2013). Collective cell migration of epithelial and mesenchymal

cells. *Cell. Mol. Life Sci.* **70**, 3481–3492.

Theveneau, E., Marchant, L., Kuriyama, S., Gull, M., Moepps, B., Parsons, M. and Mayor, R. (2010). Collective Chemotaxis Requires Contact-Dependent Cell Polarity. *Dev. Cell* **19**, 39–53.

Trepat, X., Chen, Z. and Jacobson, K. (2012). Cell migration. *Compr. Physiol.* **2**, 2369–92.

Uemura, O., Okada, Y., Ando, H., Guedj, M., Higashijima, S.-I., Shimazaki, T., Chino, N., Okano, H. and Okamoto, H. (2005). Comparative functional genomics revealed conservation and diversification of three enhancers of the isl1 gene for motor and sensory neuron-specific expression. *Dev. Biol.* **278**, 587–606.

Valiente, M. and Marín, O. (2010). Neuronal migration mechanisms in development and disease. *Curr. Opin. Neurobiol.* **20**, 68–78.

Villar-Cerviño, V., Molano-Mazón, M., Catchpole, T., Valdeolmillos, M., Henkemeyer, M., Martínez, L. M., Borrell, V. and Marín, O. (2013). Contact repulsion controls the dispersion and final distribution of Cajal-Retzius cells. **77**, 457–471.

Vivancos, V., Chen, P., Spassky, N., Qian, D., Dabdoub, A., Kelley, M., Studer, M. and Guthrie, S. (2009). Wnt activity guides facial branchiomotor neuron migration, and involves the PCP pathway and JNK and ROCK kinases. *Neural Dev.* **4**, 7.

Volk, T. and Geiger, B. (1984). A 135-kd membrane protein of intercellular adherens junctions. *EMBO J.* **3**, 2249–2260.

Wacker, A. and Gerhardt, H. (2011). Endothelial development taking shape.

Wada, H. (2005). Dual roles of zygotic and maternal Scribble1 in neural migration and convergent extension movements in zebrafish embryos. *Development* **132**, 2273–2285.

Wada, H., Iwasaki, M., Sato, T., Masai, I., Nishiwaki, Y., Tanaka, H., Sato, A., Nojima, Y. and Okamoto, H. (2005). Dual roles of zygotic and maternal Scribble1 in neural migration and convergent extension movements in zebrafish embryos. *Development* **132**, 2273–85.

Wada, H., Tanaka, H., Nakayama, S., Iwasaki, M. and Okamoto, H. (2006a). Frizzled3a and Celsr2 function in the neuroepithelium to regulate migration of facial motor neurons in the developing zebrafish hindbrain. *Development* **133**, 4749–4759.

Wada, H., Tanaka, H., Nakayama, S., Iwasaki, M. and Okamoto, H. (2006b). Frizzled3a and Celsr2 function in the neuroepithelium to regulate migration of facial motor neurons in the developing zebrafish hindbrain. *Development* **133**, 4749–59.

Waites, C. L., Craig, A. M. and Garner, C. C. (2005). Mechanisms of Vertebrate Synaptogenesis. 251–276.

Wallingford, J. B. (2006). Planar cell polarity, ciliogenesis and neural tube defects. *Hum. Mol. Genet.* **15**, 227–234.

Wallingford, J. B. (2012). Planar cell polarity and the developmental control of cell behavior in vertebrate embryos. *Annu. Rev. Cell Dev. Biol.* **28**, 627–53.

Walsh, G. S., Grant, P. K., Morgan, J. a and Moens, C. B. (2011). Planar polarity pathway and Nance-Horan syndrome-like 1b have essential cell-autonomous functions in neuronal migration. *Development* **138**, 3033–42.

Wanner, S. J. and Prince, V. E. (2013a). Axon tracts guide zebrafish facial branchiomotor neuron migration through the hindbrain. **915**, 906–915.

Wanner, S. J. and Prince, V. E. (2013b). Axon tracts guide zebrafish facial branchiomotor neuron migration through the hindbrain. *Development* **140**, 906–15.

Wanner, S. J., Saeger, I., Guthrie, S. and Prince, V. E. (2013). Facial motor neuron migration advances. *Curr. Opin. Neurobiol.* 1–8.

Weber, G. F., Bjerke, M. A. and DeSimone, D. W. (2012). A Mechanoresponsive Cadherin-Keratin Complex Directs Polarized Protrusive Behavior and Collective Cell Migration. *Dev. Cell* **22**, 104–115.

Westerfield, M. (2000). *The zebrafish book. A guide for the laboratory use of zebrafish (Danio*

rerio). Eugene: Univ. of Oregon Press.

Wong, G. K. W., Baudet, M.-L., Norden, C., Leung, L. and Harris, W. a (2012). Slit1b-Robo3 signaling and N-cadherin regulate apical process retraction in developing retinal ganglion cells. *J. Neurosci.* **32**, 223–8.

Wu, W., Wong, K., Chen, J. H., Jiang, Z. H., Dupuls, S., Wu, J. Y. and Rao, Y. (1999). Directional guidance of neuronal migration in the olfactory system by the protein Slit. *Nature* **400**, 331–336.

Yap, A. S., Niessen, C. M. and Gumbiner, B. M. (1998). The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120(ctn). *J. Cell Biol.* **141**, 779–789.

Zannino, D. a, Sagerström, C. G. and Appel, B. (2012). olig2-Expressing hindbrain cells are required for migrating facial motor neurons. *Dev. Dyn.* **241**, 315–26.