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Characterizing the Cellular Nature of the Physical Interactions Necessary for Collective Neuron Migration

Rebecca Vareed
Virginia Commonwealth University

CHARACTERIZING THE CELLULAR NATURE OF THE PHYSICAL INTERACTIONS NECESSARY FOR COLLECTIVE NEURON MIGRATION

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science at Virginia Commonwealth University.

by

REBECCA D. VAREED
BACHELOR OF SCIENCE, VIRGINIA COMMONWEALTH UNIVERSITY 2017

Director: GREGORY S. WALSH, PH.D.
ASSOCIATE PROFESSOR, DEPARTMENT OF BIOLOGY

Virginia Commonwealth University
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List of Abbreviations

CAM- cell adhesion molecule

Cdc- cell division cycle

CE- Convergent extension

CIL- Contact inhibition of locomotion

Celsr- Cadherin EFG LAG seven-pass G-type receptor

Cxcr- chemokine (C-X-C motif) receptor

Daam: Disheveled-associated activator of morphogenesis

Dgo- diego

Dsh, dvl- disheveled

DCX- doublecortin

FBMN- Facial branchiomotor neuron

Fzd- frizzled

GABA- gamma-aminobutyric acid

GFP- Green fluorescent protein

GTP- Guanosine triphosphate

Hgf- hepatocyte growth factor

HGFR- hepatocyte growth factor receptor

Hox- homeobox

Isl- ISL LIM homeobox

LIS1- platelet-activating factor acetylhydrolase IB subunit alpha

MO- morpholino oligonucleuotide

NCC- Neural crest cell

Npn- neuropilin

PCP- Planar cell polarity

Pk- prickle

RFP- Red fluorescent protein

Sema- semaphorin

Scrib- scribble

SDF- stromal cell-derived factor

Tbx- T-box

Vangl- Van Gogh like

Vegf- vascular endothelial growth factor

WT- wild-type

ABSTRACT

CHARACTERIZING THE CELLULAR NATURE OF THE PHYSICAL INTERACTIONS NECESSARY FOR COLLECTIVE NEURON MIGRATION

By Rebecca D. Vareed, B.S.
A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biology at Virginia Commonwealth University.
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Major Advisor: Gregory S. Walsh, Ph.D. Associate Professor, Department of Biology

Neuronal migration is an essential process in the development of the nervous system. Neurons are born in one location and migrate sizable distances to their final location. In many other developmental processes, cells migrate as collectives, where the migration of one cell influences the migration of another cell; this process has yet to be shown in the developing central nervous system. Using the conserved tangential migration of facial branchiomotor neurons (FBMNs), I aim to determine the nature of the collective migration in the developing nervous system. Here, two models of FBMN collective migration are tested: the "Pioneer" model, where following FBMNs migrate intimately on the axon of the first neuron to migrate and the "Contact inhibition of locomotion (CIL)" model, where transient cell-cell contacts are the driving influence of the proper caudal migration of FBMNs. Using fixed tissue imaging, it was found that early born FBMNs do not contact the axon. In contrast, they are more likely to make soma-soma contact and display morphology typical of CIL. FBMNs that do contact the axon do not display an elongated morphology that is predicted of a cell using the leader axon as a substrate for migration. Further, wild-type FBMNs are able to rescue PCP-deficient FBMNs. Therefore, blastula-stage transplantation of PCP-deficient neurons into wild-type hosts allows us to live image the method of collective migration. CIL events were observed between PCP-deficient neurons and wild-type neurons, indicating that PCP is not required for CIL. In addition, PCP-deficient neurons making sustained contact with wildtype axons were not rescued, arguing against the Pioneer model. Taken together, these observations are more consistent with the "CIL" model of FBMN collective migration in which transient soma-soma interactions are required for the coordinated movement of neurons as they migrate in the developing nervous system.

INTRODUCTION

Development of the nervous system and neuron migration

The human nervous system is constructed of a complex network of neurons responsible for sensing environmental stimuli and controlling behavior. Neuron migration is an essential step in the formation of these neural circuits. Neurons are often born in one location and migrate sizable distances to their final destination where they assemble into neural circuits. Neurons integrate numerous cellular and molecular cues to navigate to their final positions. These include heterotypic cell-cell interactions with neuroepithelial cells, homotypic interactions with neighboring neurons, intrinsic factors like polarity complexes and transcription factors, and responses to extrinsic factors, such as secreted chemotactic factors.

Mutations in genes that regulate neuron movement have been shown to cause rare neurodevelopmental disorders such as epilepsy, Tourette's syndrome, and lissencephaly, as well as more common neurodevelopmental disorders such as autism and schizophrenia (*Ross et al., 2002; Valiente et al., 2010*). For example, mutations in DCX, implicated in regulating microtubule organization and stability, and LIS1, implicated in regulation of microtubule organization and dynein motor function, are responsible for the majority of classical lissencephaly (*Ross et al. 2002)*. Mutations in reelin, an extracellular matrix protein that regulates cell-cell interactions necessary for proper cell positioning, result in cerebellar hypoplasia (*Ross et al., 2002)*. It is therefore essential to understand the cellular and molecular mechanisms of neuron movement in the developing nervous system.

Neurons migrate radially or tangentially depending on where they are born and their final destination. Radial migration occurs when cells migrate from the ventricular zone towards the surface of the brain. Neurons that migrate radially are initially born from radial glia (neural progenitors of the cortex) from asymmetric cell division. Subsequently, radially migrating neurons re-attach with radial glial fibers, using them as a substrate for migration towards the marginal zone. As they migrate radially, these neurons make intimate contact with radial glial fibers as they migrate to the developing cortical plate, displaying an elongated bipolar morphology, with a thick leading process and a thin trailing process (*Kriegsten et al., 2004)*. These leading and trailing processes are tightly associated with and adhered to the glial fiber as the cell migrates. Cell adhesion molecules (CAMs) such as N-cadherin, a calcium-dependent homotypic CAM, play an important role in neuron-radial glial cell migration. Neuron-specific inactivation of N-Cadherin leads to a defect in radial migration and an increase in the distance between neuron and radial glial fiber (*Kadowaki et al, 2007; Kawauchi et al, 2010*).

Tangentially migrating neurons, unlike radially migrating neurons, move through the parenchyma, perpendicular to radial glia, to reach their final destination. Tangentially migrating neurons rely on integration of repulsive and/or attractive chemotactic cues in the environment, rather than contact with radial glia, to navigate to their final destination (*Marin et al., 2003; Evsyukova et al., 2013*). There are several examples of tangential neuron migration, including the migration of GABAergic inhibitory precursors neurons from the ganglionic eminences to the cortex (*Anderson et al, 1997; Tamamaki et al., 1997*). These precursors rely on semaphorin-neuropilin interactions to migrate to the cortex. The repulsive cues established by Sema3 guides proper dorsal migration by Npn-1 positive cells, while also ensuring that cells do not migrate improperly into ventricular zone (*Tammamaki et al., 2003*). Another example of tangential

migration is the posterior/caudal movement of the facial branchiomotor neurons in the hindbrain (see below for more information).

Cell migration

Cell migration involves front-rear polarity, actin polymerization, and membrane dynamics. In general, cells develop an intrinsic polarity that consists of a front, where membrane protrusions such as lamellipodia and filopodia, and a rear, where cell membrane is contracted, and adhesions are regulated to detach as the cell advances. Most migrating cells have the nucleus, Golgi apparatus, and microtubule organizing center arranged in a line pointing towards the front axis of the cell. This allows for cargo from the Golgi to be transported along microtubules to the front of the cell as it migrates. This intrinsic polarity may be influenced by multiple cues including cell-cell interactions, cell-matrix interactions, and secreted factors.

Morphologically, front-rear polarity is characterized by the presence of a large lamellipodium and smaller, dynamic filopodia. Lamellipodia and filopodia are stabilized at the leading edge of the migrating cell, and the cell exhibits motility through a protrusion, adhesion, contraction, and retraction cycle. This polarity is established by activation of small Rho family GTPases, such as Cdc42 and Rac1 at the front of the cell and RhoA at the rear of the cell, which regulate actin dynamics and protrusion formation (*Takai et al, 2001; Jaffe & Hall 2005*). Rac1 typically promotes branched actin polymerization seen in lamellipodia, cdc42 promotes long parallel actin polymerization in filopodia, and RhoA promotes actin-myosin contraction and/or the dis-assembly of actin filaments at the rear of the cell (*Nyugen et al., 2016*). In the absence of directive cues, migrating cells display random motility *(Bard and Hay 1975). In vitro*, cells

display peripheral lamellipodia, mostly in part due to high activation of Rac at the leading edge (*Pankov et al., 2005*).

In order to migrate directionally, cells require cues such as contact-guidance, cell-cell contact, or chemotactic gradients (*Rieg et al., 2014*). When given a directional cue, protrusion formation stabilizes along a leading axis, allowing cells to exhibit proper directional movement. These extrinsic cues ultimately affect the activation Rac and cdc42 at the front and RhoA activation at the rear of the cell, leading to polarized directional movement. Inhibition of random protrusion formation is mediated by Rho, as well as membrane tension, which acts as a physical signal to inhibit protrusion formation anywhere but the leading edge (*Rieg et al., 2014*). Multiple extrinsic cues can regulate the activity of Rac and Rho, and hence actin polymerization and de-polymerization, leading to guided movement of cells.

While individual cues can be isolated and studied *in vitro*, neuron migration *in vivo* relies on multiple cues found in the 3-dimensional milieu through which they navigate. These include cell-cell contact, contact guidance, and chemotactic gradients may interact. Importantly, many cell types *in vivo* often migrate as collective groups (*Rieg et al., 2014*).

Collective cell migration

Collective cell migration is defined as the coordinated and cooperative movement of a cell population. Importantly, the behavior and migration of one cell can influence the migratory behavior of another cell. Collective cell migration is borne from cell-cell interactions, where cooperation between neighboring cells contributes to their overall directionality. These may range from stable physical links between cells in a group (e.g. epithelial cells) to transient interactions between neighboring cells (e.g. mesenchymal cells, neural crest cells (NCC)). Given

the necessity of cell-to-cell contact in collective migration, as contact events are critical for collective migration, it is not surprising that cell adhesion molecules play a central role. For example, during germ layer morphogenesis in the course of gastrulation, lateral line migration in zebrafish, and epithelial sheet migration during wound healing, cells remain physically connected throughout their migratory path (*Olson et al., 2018; Danjo et al., 1998*). This form of collective migration has also been described in invasive carcinomas (*Yamamoto et al., 1983; DiCostanzo et al., 1990; Gaggioli et al., 2007*). In this instance, the leading cells physically pull other cells behind them as they migrate, bound stably by physical cell-cell connections. These physical cell-cell connections are responsible for migration of the cell population; for example, E-cadherin is essential for proper migration of epithelial sheets in wound healing, as loss of E-cadherin halts migration entirely (*Li et al., 2012)*.

Cells can migrate collectively as individuals, making transient contacts throughout migration. This occurs when cells migrate as loose chains or streams, which has been best described in the migration of neural crest cells (NCC) that migrate as a stream of cells (*Sadaghiani and Thiebaud, 1987*). Individual cells make transient contact with neighboring NCCs, and evidence suggests these transient contacts are essential for NCC directional migration (*Theveneau et al., 2010*). For instance, when NCCs are placed in a gradient of an attractive chemotactic factor, NCCs only migrate directionally if there is a large enough density of NCCs to promote NCC-NCC interactions. If NCCs are placed as a lone cell within this gradient, they often wander randomly and without persistent directed movement (*Theveneau et al., 2010*). These studies indicate that cell-cell contact is necessary for NCCs to polarize and respond correctly to other extrinsic cues in the environment. Interestingly, cadherins, a family of calcium-dependent cell adhesion molecules, are thought to play an important role as key regulators of

cell-to-cell communication that allow cells to polarize and collectively migrate with high directionality. In the absence of N-cadherin, NCCs migrate randomly even in the presence of a chemotactic gradient (*Theveneau et al., 2010*).

Although studies of collective migration in 1D or 2D culture have yielded valuable insights, the number of model systems to study collective migration of streams or chain migration *in vivo* are few. The study of neural crest cells in frog and zebrafish have provided valuable insights into the cellular and molecular mechanisms of collective cell migration. A second model relies on the collective migration of hemocytes in Drosophila that require cell-cell contact to disperse evenly across the organism (*Stramer et al., 2010*). Similarly, Cajal-Retzius cells make transient interactions to ensure an even distribution as they migrate over the surface of the cortex (*Villar-Cervino et al., 2013*). However, the study of collective migration in the developing nervous system has not been characterized. There is a need to develop additional model systems *in vivo* to identify the molecular mechanisms of collective migration, especially in the central nervous system.

Contact inhibition of locomotion

How do transient cell-cell interactions lead to directional movement? An observation of cells undergoing transient collisions by Abercrombie suggested that cell-cell contact leads to an arrest in migration followed by repolarization and migration in the opposite direction, leading to dispersal of the cells (*Abercrombie & Heaysman, 1953; Abercrombie & Ambrose, 1958*). This behavior of the cells has been coined contact inhibition of locomotion. Contact inhibition of locomotion (CIL) occurs when a cell contacts another cell, ceases its movement, collapses protrusions at the site of contact, repolarizes with protrusive behavior initiating on the free

surface of the cell (away from the site of contact), and changes its directional migration. CIL should be thought of as a repulsive influence on neighboring cells, thereby leading to dispersion of the collective. CIL has been demonstrated to be the driving force behind the collective cell migration of neural crest cells and Cajal-Retzius cells in vertebrates (*Becker et al., 2013; Villar-Cervino et al., 2013)*. CIL has also been shown to show an important role in the collective migration of hemocytes in Drosophila (*Stramer et al., 2010*).

CIL is mediated by transient cell surface interactions through classical signaling molecules. In neural crest cells, cadherins are necessary for proper CIL, while Eph-Ephrin interactions mediate the repulsive CIL behavior of both cancer cells and Cajal-Retzius cells (*Becker et al, 2013; Batson et al., 2013; Villar-Cervino et al., 2013).* Migrating fibroblasts rely on Slit-Robo interactions at collision sites to properly engage in CIL (*Roycroft et al., 2016*). Engagement of these adhesion molecules and transmembrane receptors leads to repolarization and changes in protrusion dynamics by modulating the Rac-Rho axis in the relevant cell types.

N-cadherin has been shown to be necessary for proper migration in neural crest cells, as inhibition of N-cadherin signaling results in failure of neural crest cell migration (*Theveneau et al., 2010*). Importantly, N-cadherin is essential for CIL, as N-cadherin deficient cells display cell-cell overlapping, as well as making overlapping protrusions between neighboring cells. Wild-type cells display clear cell-cell boundaries and do not make overlapping protrusions. Further, N-cadherin deficient cells do not display the halt in migration and change in behavior that wild-type cells display after a collision event. This suggests that cells lacking N-cadherin have lost the ability to inhibit protrusions, and therefore have lost CIL behavior that is necessary for them to migrate properly (*Theveneau et al., 2010)*.

Planar cell polarity

Planar Cell Polarity (PCP) refers to the coordinated alignment of cells within the epithelial plane. PCP signaling involves the evolutionarily conserved core transmembrane proteins Frizzled (Fzd), Celsr, Van Gogh-like (Vangl), and the cytoplasmic proteins Disheveled (Dsh), Prickle (Pk), and Scribble (Scrib), which localize asymmetrically to regulate polarity. PCP has been extensively described in *Drosophila* wing epithelial cells. These wing cells are characterized by trichomes, an actin-based hair that is localized to the distal side of each wing cell. The aforementioned core PCP proteins are responsible for the localization of this trichome, as mutations in these genes result in improper wing hair organization (*Vinson & Adler, 1987*; *Krasnow et.al. 1995*, *Shimada et al., 2001*; *Tissir & Goffinet, 2013*). The proper orientation of trichomes is established by the asymmetrical localization of Fzd, Celsr, Dsh, and Dgo to the distal side and Vangl, Celsr, and Pk to the proximal side of each wing cell (*Strutt 2001; Montcouquiol et al., 2006; Strutt & Strutt, 2006*).

This asymmetrical localization of core PCP proteins is highly conserved and has been implicated in important developmental processes in vertebrates as well. PCP is also responsible for the proper alignment of epithelial cells. For instance, loss of any PCP core protein leads to a randomized alignment of hair cells in the inner ear, misaligned hair follicles in the skin, and loss of asymmetric localization of mono-cilia in floorplate cells, responsible for the circulation of cerebrospinal fluid (CSF) (*Borovina et al., 2010; Walsh et al., 2011*). The asymmetry in morphology is also mirrored by an asymmetry in the distribution of core PCP proteins. For instance, Vangl2 protein is found on the same side as the cilia in inner ear hair cells, whereas Fzd3 is found localized to the opposite cell membrane (*Borovina et al., 2010*; *Wallingford 2012*).

In addition to its role in static epithelial cells, PCP signaling plays a role in the movement and migration of various cell types. Perhaps the best studied example is convergent extension movements during gastrulation of vertebrate embryos. Convergent extension (CE) is an essential process for body axis elongation, where cells must intercalate and narrow along one axis leading to extension of the body axis. This process was first described in *Xenopus* but has also been described in Zebrafish gastrulation (*Keller et al., 1988; Heisenberg et al., 2012*). PCP has been implicated in the regulation of convergent extension, as loss of Wnt11 signaling results in improper CE (*Ulrich et al., 2005*). Further, many PCP mutants, such as *vangl, dsh, pk1a,* and *scrib*, display a convergent extension defect (*Tada et al., 2012*). PCP signaling is also necessary for other types of cell migration including wound healing, neural crest migration, and neuron migration (see below).

PCP functions further in many processes in the development of the nervous system. First, PCP is essential for proper neural tube closure. PCP signaling has been implicated in the proper movement and intercalation of dividing cells during neural tube closure, and when interrupted can lead to defective midline establishment and failure of neural folds to fuse (*Ciruna et al., 2006; Ybot-Gonzales et al., 2007).* PCP is also essential for axon pathfinding, as *fzd*, *vangl*, *celsr3*, and *scrib* are required for proper pathfinding of spinal cord commissural neurons, as well as dopaminergic neurons in the brainstem (*Sun et al., 2016; Zou, 2004; Tissir and Goffinet, 2013*). PCP core components have also been shown to be required for the posterior tangential migration of facial branchiomotor neurons in the developing hindbrain (see below).

Migration of facial branchiomotor neurons

The migration of facial branchiomotor neurons (FBMNs) in zebrafish is highly stereotyped and provides an excellent model system to investigate the cellular and molecular mechanisms of neuron migration. FBMNs are a subset of cranial motor neurons that are born in rhombomere 4 at 16 hpf and undergo a tangential migration caudally to reach r6/r7 in zebrafish by 48hpf (Figure 1). This migration is evolutionarily conserved in mammals, fish, and lizards (*Chandrasekhar, 2004*). FBMNs are born ventrally and migrate along the ventral aspect of the hindbrain. During the course of their migration, FBMNs come in contact with several different cell types, and these cell-cell interactions can provide guidance cues to promote proper caudal movement. FBMNs interact homotypically with other FBMNs, as well as heterotypically with the surrounding neuroepithelial cells.

As FBMNs migrate, they trail their axons behind as they move caudally (*Chandrasekhar, 2004*). FBMN axons exit the spinal cord from r4 (where they are born), where they make up the motor portion of the facial nerve (cranial nerve VII). In humans, FBMNs innervate muscles responsible for facial expression, whereas in fish, FBMNs innervate muscles associated with some aspects of jaw movement, as well as muscles that move the operculum that covers the gills (*Chandrasekhar, 2004*).

Previous experiments have identified some of the proteins responsible for regulation of proper FBMN migration. For instance, the transcription factor Hoxb1a is expressed in r4, and is essential for proper patterning including differentiation of FBMNs, as they are born in r4 (*McClintock et al., 2002)*. Mutation or knockdown of *hoxb1a* in either mouse or zebrafish leads to a complete block in FBMN caudal migration (*Rohrschneider et al., 2007; Gavalas et al.,*

2003). Further, *tbx20*, another transcription factor, is expressed by cranial motor neurons and is essential for proper caudal FBMN movement (*Song et al., 2006*).

Secreted chemotactic factors are also responsible for the proper caudal migration of FBMNs. SDF1, Vegf164, and Hgf1/2, and their obligate receptor molecules such as Cxcr4, HGFR, and Met have all been implicated in the regulation of FBMN movement (*Sabede et al, 2005; Schwarz et al. 2004; Elsen et al., 2009; Cubedo et al., 2006).* Extracellular matrix proteins expressed at the base of the neural tube, such as Laminin, also play a role in mediating proper migration (*Grant & Moens, 2010*; *Sittaramane et al., 2009*). The evolutionarily conserved planar cell polarity (PCP) pathway has also been specifically implicated in regulating the caudal migration of FBMNs.

Planar cell polarity and facial branchiomotor neurons

PCP is essential for the regulation of proper caudal migration of FBMNs. Mutations in the core PCP genes *fzd3a*, *celsr1/2*, *vangl2*, and *scrib* result in the aberrant stalling of FBMN migration in r4 (*Wada et al, 2005,2006; Jessen et al., 2002; Glasco et al, 2012*). Through generation of chimeric embryos via gastrula stage transplants, it has been shown that these essential PCP proteins function non-cell autonomously. That is, wild-type cells transplanted into a PCP-deficient environment are unable to migrate properly (*Wada et al., 2005, 2006; Davey et al., 2016; Jessen et al., 2002; Sittaramane et al., 2013; Walsh et al., 2011).* This suggests that PCP-dependent polarization of the neuroepithelial environment is necessary for proper migration of FBMNs.

Evidence suggests that the PCP protein Pk1b is not required non-cell autonomously (*Rohrschneider et al., 2007; Mapp et al., 2011*). *Pk1b*, unlike other PCP genes that are expressed ubiquitously, is only expressed in FBMNs as they move from r4 through r6 and not in the surrounding neuroepithelial cells (*Rohrschneider et al., 2007*). Absence of *pk1b* expression results in failure of FBMNs to migrate out of r4 (*Rohrschneider et al., 2007; Mapp et al., 2011*). Unlike other PCP mutants, wild-type cells transplanted into a *pk1b*-deficient host are able to properly migrate posteriorly (*Mapp et al., 2011; Walsh et al., 2011)*. This indicates that the neuroepithelial environment in a *pk1b*-deficient host retains the proper polarization necessary for proper caudal directionality of migrating FBMNs. Further, reciprocal transplants demonstrate that *pk1b*-deficient cells are unable to migrate in a wild-type host (*Rohrschneider et al., 2007*). This supports a cell autonomous role for Pk1b in FBMN migration.

It has been demonstrated that other core PCP proteins function non-cell autonomously (*Wada et al., 2005, 2006; Davey et al., 2016; Jessen et al., 2002; Sittaramane et al., 2013; Walsh et al., 2011*). If other PCP proteins function within the neurons cell autonomously, then it would be predicted that a PCP mutant FBMN would fail to migrate in a wild-type host. However, after analysis of chimeric embryos, it was observed that while a large number of PCP-mutant neurons fail to migrate, approximately half of *fzd3a-, vangl2-,* or *scrib*-deficient FBMNs did migrate appropriately when transplanted into a wild-type host (*Wada et al., 2006; Walsh et al., 2011; Davey et al., 2016*). The reason for this is elucidated by the novel transplantation of *fzd3a, vangl2,* and *scrib* mutant donor cells into *pk1b* mutant hosts. In *pk1b* mutant hosts, the environment is wild-type, as previously discussed, but host neurons fail to migrate (Figure 2). Under these conditions, PCP-deficient donor cells completely fail to migrate caudally, remaining blocked in r4 with the unmigrated host neurons (*Walsh et al., 2011; Davey et al., 2016)*.

Therefore, it can be concluded that the only reason some PCP-deficient neurons can migrate in a wild-type environment is due to the presence of properly migrating wild-type neurons. That is, wild-type neurons can rescue the posterior migration of an adjacent PCP-deficient cell.

First, this observation demonstrates that core PCP proteins function cell autonomously (within the neuron) as well as within the environment (non-cell autonomous) to regulate caudal movement. Secondly, this observation suggests that one neuron can influence the migration of a neighboring neuron, and therefore FBMN migration should be classified as a collective migration. This was the first demonstration of collective behavior by neurons migrating in the developing central nervous system. It should be noted that the observation of collectiveness only occurs in mosaic embryos generated by cell transplantation that is easily accomplished in zebrafish embryos.

The molecular mechanism regulating the collective migration of FBMNs is still being elucidated. However, given that a wild-type neuron can rescue the migration of a PCP-deficient neuron, it appears that the neuron-neuron interactions that drive and promote collective movement are independent of PCP-signaling (at least in the rescued cell). Given that other collective migrations are mediated by cell-cell interactions and cell adhesion molecules, it was postulated that a cell adhesion molecule is responsible for neuron collective migration. Indeed, inactivation of N-cadherin specifically within FBMNs leads to a loss of collective neuron migration (*Rebman et al., 2016*). Specifically, in mosaic embryos, wild-type neurons are unable to rescue N-cadherin-deficient FBMNs (*Rebman et al., 2016*). These results support a model in which the collective cell behaviors of FBMNs are driven by N-cadherin-based neuron-to-neuron

interactions. It remains unclear what the nature of the physical interactions are that drive collective neuron movement.

Neuron-to-neuron interactions clearly play an important role during FBMN migration; however, no coherent model currently exists for how neuron-to-neuron cell contacts contribute to sustained directed migration of FBMNs. *Based on available literature, I propose two alternative cellular models to explain how neuron-to-neuron contacts may drive collective migration of FBMNs.*

1) The Pioneer Model:

It was proposed that the very first FBMN to migrate caudally is a "pioneer" neuron that trails an axon behind it as it migrates (*Wanner et al., 2013*). In this model, "follower" neurons use Cdh2 to control neuronal attachment to the trailing pioneer axon and promote caudal migration along a preferred substrate. This would indicate a soma-axon interaction model as the mode of migration of FBMNs. Support for this model derives from experiments in which laser ablation of the leading neuron's axon results in failure of some late-born follower FBMNs to properly migrate out of r4 (*Wanner et al., 2013*). This model postulates that similar to the radial migration of neurons in the developing cortex, all tangentially migrating FBMNs adhere to the leader axon as a necessary substrate for migration. A prediction of this model would suggest that FBMNs make intimate contact with the pioneer axon and may adopt an elongated morphology as they crawl along the Pioneer axon. Intimate contact is defined here as a following cell displaying an elongated morphology similar to migrating neurons on radial glia fibers, (*Viskari, 2019*). That is, the migrating cell will be elongated and display two opposing protrusions that are tightly bound to or wound around the leader axon anterior and posterior to the migrating cell's soma.

This model 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 a wild-type Pioneer axon substrate to move in the caudal direction (Figure 3). In this model of rescue, it is expected that all PCP-deficient FBMNs that contact a wild-type axon would rescue and migrate properly along the length of the leader axon.

2) The CIL Model:

An alternative hypothesis suggests that N-cadherin-mediated neuron-neuron interactions lead to (CIL) behaviors. Preliminary evidence from the Walsh lab supports a soma-soma interaction model (*Rebman et al., data not shown)*. Live imaging studies indicate that wild-type FBMNs exhibit CIL behavior (Figure 4). That is, as FBMNs migrate, they often collide into their neighbor. This collision between two neighboring FBMNs often leads to a halt in migration followed by a reversal in direction of migration of the follower/trailing neuron.

The CIL model could explain the collective migration "rescue" of PCP-deficient FBMNs if wild-type FBMNs re-polarize PCP mutant neurons via Cdh2-based soma-to-soma contact. In this scenario, a wild-type neuron induces polarized protrusive behavior in PCP-deficient neurons towards the caudal direction; these protrusions may be stabilized along the posterior free edge by an as-yet-unidentified chemotactic extrinsic cue. This model suggests that PCP-deficient neurons migrate in front of a wild-type neuron that can continue to polarize the neuron in the proper direction via CIL inducing collisions. In the absence of continuous collisions that encourage caudal directionality, not every neuron may be rescued.

While both of these models support the notion that N-cadherin mediated neuron-neuron interactions are necessary for the proper migration of FBMNs, it is still unclear which mode of neuron-neuron contact is occurring in migrating FBMNs. As previously described, collective migration has been observed via the N-cadherin mediated rescue of PCP-deficient FBMNs by wild-type cells. In order to fully understand the mechanism of collective migration of FBMNs, it is essential to determine whether FBMN migration is influenced by soma-axon contact (Pioneer Model) or soma-soma contact (CIL Model).

Research Aim

It is essential to determine by which mechanism FBMNs are undergoing collective migration in order to fully characterize this migration process. The aim of this study is to determine the nature of the physical interactions that mediate the collective migration of FBMNs. It is important to note that neither model is mutually exclusive. In this study, I have utilized FBMNs as a model to determine the nature of cell-cell contacts that mediate collective migration in the developing nervous system.

Significance

The experiments conducted here explore the cellular mechanism of neuron migration. Using a combination of anatomy, mosaic analysis, chimeric analysis, and live cell imaging, I have described the cellular interactions that drive the collective behavior of neurons as they migrate. The significance of observing collective migration in the developing nervous system cannot be overstated. Further insight into the underlying mechanisms through which cell-cell interactions drive directed cell movements will aid in future efforts aimed at devising therapeutic

strategies to ameliorate the condition of children born with neuron migration defects that lead to more serious neurodevelopmental disorders.

MATERIALS AND METHODS

Fish strains

Zebrafish (Danio rerio) were maintained according to standard procedures and were staged as previously described (C.B. Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). The *pk1b* mutant was originally described as *pk1b*^{fh122} (Mapp et al., 2011). *Tg(islet1:GFP)rw0* fish were originally described by Higashijima et al, 2000. *Tg(islet1:memb-mRFP1)* was originally described as *Tg(zCREST1:memb-mRFP1)* (*Grant & Moens, 2010*). *Tg(islet1:GFP-CaaX)* was made in the Walsh lab facility (*Jane Rebman, unpublished*).

Microinjections

Injection dishes were prepared with 1.2% agarose in fish water using a float mold to create triangular wells with a 45° angle as a backstop for injections. Glass capillary needles were pulled using a P-97 Flaming/Brown Micropipette puller (Sutter Instrument). Zebrafish were placed in crossing cages with a removable divider to separate males and females. Dividers were pulled to allow fish to mate and embryos were collected and placed in injection dishes. DNA encoding the *isl1:GFP-CaaX* plasmid (50 ng/uL) was co-injected with Tol2 transposase mRNA (50 ng/uL) into *Tg(isl1:memb-RFP)* embryos at the 1 cell stage. The dish was positioned on the stage such that as the needle enters the embryo, the embryo is pushed against the back of the well. The loaded injection needle is entered into the embryo, taking care to inject into the center of the embryo. Embryos were removed from injection dishes and placed in petri dishes containing fish water and incubated at 28.5°C until the desired stage.

Morpholino Injections.

Tg(isl1:GFP) embryos were collected and placed in injection dishes, as above. Embryos were injected with 1 nL of a cocktail containing 2.5ng/uL of *pk1b* I3E4 MO (5'-GGCAGTAGCGAATCTGTGTTGAAGC-3') and 2.5ng/uL of *pk1b* E6I6 MO (5'-TTAATGAAACTCACCAATATTCTCT-3'). All injections were done with an ASI MPPI-3 (Applied Scientific Instrumentation) pressure injector. Embryos were positioned and injected as above. Embryos were removed from injection dishes and placed in petri dishes containing fish water and incubated at 28.5°C until the desired stage.

Blastula Stage Transplants

The apparatus used for cell transplantation in the zebrafish blastula consists of a micrometer drive-controlled Hamilton syringe attached by a three-way stopcock to a reservoir of mineral oil and to a micropipette holder through a length of flexible tubing. After assembling the transplantation rig, it is filled with mineral oil, taking care to eliminate all air bubbles from the system. The positioning of the micropipette holder and needle is controlled by a Narishige manual micromanipulator.

Transplantation needles were made from glass capillary pipettes and pulled using a P-97 Flaming/Brown Micropipette puller (Sutter Instrument. The tip of the needle was broken off under a dissecting microscope using a straight edge razorblade at the point where the inner diameter of the needle is slightly larger than the cells to be transplanted, approximately 50-60 mm for blastula stage transplants. Needles were then fire polished using a MF-200 Microforge (World Precision Instruments).

Injection dishes were prepared with 1.2% agarose in fish water using a float mold with rows of wedge-shaped protrusions in a Petri dish. Once the mold is removed, it leaves an agar mold that contains rows of triangularly shaped wells each just large enough to hold one embryo. Blastula stage embryos were enzymatically dechorionated with pronase and were individually loaded into each well with a fire polished glass pipette such that the donor embryos are placed down one column and the host embryos are placed down the adjacent column. Embryos were positioned in the transplant wells on their sides with yolk pointing upwards.

The dish was positioned on the stage such that as the needle enters an embryo, the embryo is pushed against the back wall of its well. Once the donor embryo was positioned properly, and the needle is gently entered into the blastula cap of the embryo. Donor cells were drawn slowly drawn up the needle, as taking cells up too quickly can cause shearing. After the desired number of cells is taken up, the pressure is reversed slightly to stop suction and the needle is removed from the embryo. The transplant dish is moved to bring the host embryo into position, and the host embryo was gently repositioned if necessary, using the transplantation needle. Donor cells are then expelled into the host embryo with extra care not to puncture the YSL or introduce a large amount of mineral oil.

After cell transplantation was complete, donor-host pairs were transferred from the mold to agarose-coated dishes to develop further.

Immunocytochemistry & immunofluorescence

Primary antibodies used were rabbit anti-GFP (Invitrogen) and mouse anti-RFP (Clonetech). Secondary antibodies used were goat anti-rabbit Alexa 488 (ThermoFisher) and

goat anti-mouse Alexa 547 (Thermofisher). Primary antibodies were used at 1:1000, secondary antibodies were used at 1:200.

Embryos were manually dechorionated and fixed at 24 and 42 hours post fertilization in 4% paraformaldehyde in 1X PBS overnight at 4°C, and then washed with PBST (1xPBS + 0.25% Triton-X. Embryos were permeabilized with ice-cold acetone. Embryos were washed with PBST before being incubated with blocking solution (PBST + 10% goat serum + 4% BSA) for one hour at room temperature. Primary antibody was added to embryos diluted in block overnight at 4°C. Embryos were then washed in PBST before secondary antibody diluted in block was added overnight at 4°C. Embryos were washed 5x in PBST for 30 minutes each. Embryos were successively dehydrated in 25%, 50%, and 75% glycerol in 1× PBS.

Embryo mounting

After immunocytochemistry and immunofluorescence, yolks were removed using sharpened tungsten wire and embryos were flat mounted on coverslips and surrounded with 75% glycerol. For live image analysis, embryos were manually dechorionated, anesthetized with Tricaine, and mounted in 1.2% low melt agarose on a glass bottomed dish.

Confocal microscopy

All images were taken using a Carl Zeiss spinning disc confocal microscope at 63X magnification. The embryos fixed in 4% PFA were deyolked and mounted laterally in 75% glycerol on a glass slide with a coverslip.

RESULTS

Early born FBMNs do not make intimate contact with the leader axon

The earliest FBMNs are born in r4 at 16 hours post fertilization (hpf) and begin their posterior migration shortly after. After the earliest FBMNs begin to migrate caudally out of r4, FBMNs continue to arise in r4 until approximately 24 hpf. It is still unclear what the nature of the neuron-neuron physical interactions are that promote the collective behavior of FBMNs. The "Pioneer Model" proposes that newly born FBMNs make soma-axon interactions to migrate out of r4, migrating on the axon left behind by the pioneer neuron. On the other hand, the "CIL" model favors soma-soma contact as the cellular basis for collective movement.

Therefore, I first sought to determine whether FBMNs are more likely to make soma-axon or soma-soma physical interactions as they begin to migrate. To accomplish this, I utilized *Tg(isl1:GFP-CaaX)* and *Tg(isl1:memb-RFP)* fish, which, under the *islet-1* promoter, allow visualization of the cranial motor neurons. These transgenic lines express a fluorescent protein tagged with a membrane-localization motif and therefore the fluorescent proteins allow specific visualization of the membrane of cranial motor neurons. This allows for analysis of the protrusive activity of cranial motor neurons. Using fixed timepoints and immunostaining, I examined the early interactions and morphology of FBMNs.

First, I examined whether early migrating FBMNs are contacting the axon of the pioneer neuron. FBMNs were observed both overlying the leading axon as well as appearing to migrate in free space near the axon. Cells that are making contact with the leading axon, however, do not appear to be intimately associated with the axon, instead overlaying it (Figure 5A-B, 4J). As previously discussed, an intimate association with the axon is similar to that of migrating

neurons on radial glia fibers, with migrating cells elongated and closely associated with the leader axon. No intimate association between the migrating follower FBMNs and the leader axon were observed. The impression of FBMNs overlying the axon were observed in projections of z-stack confocal images. However, examination of all optical sections indicated that the FBMNs were in close proximity, but not using the axon as a substrate. Protrusive activity was not localized and tightly adhered to the leader axon. Moreover, the FBMNs did not display an elongated morphology, as might be predicted if the cell was exclusively using the axon as a substrate to navigate caudally. Rather, the FBMNs were largely spherical. Other times, protrusion from a FBMN would cross over the pioneer axon, but the cell soma was located a short distance away from the pioneer axon. Further, I did observe axon-axon physical interactions between FBMNs. That is, axonal fasciculation was seen, where trailing axons of migrating FBMNs begin to adhere to each other and bundle into a single axon tract, involving both the leading cell and later born FBMNs. This typically occurs between the axons of two migrating cells, resulting in the two cells appearing very close together. However, while the cell somas of these FBMNs appear close together, there is distinct space between the soma of the following cell and the axon of the leading cell (Figure 5H, 4I).

I also observed neurons migrating independently of the leading axon, displaying polarized protrusive activity into posterior free space (Figure 5B, 4G). Similarly, large groups of cells appearing to contact each other but separated from the pioneer axon were observed (Figure 5C-E). Some of these cells may appear to make contact with the axon, again either overlaying the axon or making contact that cannot be defined as an intimate association as discussed previously (Figure 5A-B).

Quantification of the number of FBMNs making soma-axon contacts, irrespective of whether the contact was an intimate association or a single protrusion in contact with the pioneer axon, indicated that only 20 ± 11.4% (n = 339 neurons; 26 embryos) of early born migrating FBMNs make some sort of soma-axon contact (Figure 6F-G).

FBMNs are more likely to make cell-cell contact as they migrate

Visualization of early born FBMNs can be challenging in only one transgene, as there can be too much fluorescent information to properly examine cell-cell contacts. With all FBMNs expressing one transgene, cell boundaries are challenging to distinguish, and the nature of cell-cell contacts is difficult to observe and therefore accurately quantify. In order to further elucidate the nature of the cellular contacts that facial motor neurons are making as they begin their migration, I created Tol-2 mediated transient transgenics. By injecting an *isl1:GFP-CaaX* construct into *Tg(isl1:mem-RFP)* embryos, I was able to selectively label only a few FBMNs in green, compared to all the FBMNs labeled in red. These mosaics were utilized to further characterize the nature of the cell-cell contacts that early born FBMNs make. Analysis of individual neurons labeled by GFP-CaaX confirmed impressions that few follower FBMNs make soma-axon contact with the leader axon. Cells exiting r4 often do not appear to contact the leader axon as they begin migration (Figure 6A-C). Further, in the event of soma-axon contact, cells were often overlaying the axon, absent of morphology suggesting an intimate association with the leading axon (Figure 6F). That is, no cells displaying elongated, tight associations with the leader axon were observed. On the other hand, migrating cells are observed making soma-soma contacts, easily visualized in mosaics (Figure 6D-E). Quantitation revealed that the majority, 88

± 10.9% (*n* = 339 neurons; 26 embryos), of FBMNs make some sort of soma-soma contact as they are migrating (Figure 6).

If soma-soma contact is indeed the mode of migration used by FBMNs, these contacts would be predicted to induce CIL and a collapse of protrusions at sites of contact would be observed. Indeed, in mosaic embryos, cells making soma-soma contact displayed an absence of protrusions at the site of contact (Figure 7). Similarly, colliding cells making protrusions away from the site of collision into free space were observed, which is a behavior typical of CIL (Figure 7A'-A'', 6B'). These observations suggest that not only are FBMNs more likely to make soma-soma contact than soma-axon contact, but also that these soma-soma contacts may have an influence on protrusion dynamics that subsequently alter the directionality of FBMNs.

While the majority of neurons are contacting either the soma or the axon of another cell in single snapshots during migration, there are FBMNs that are not in physical contact with another neuron; that is, they are not making any homotypic cell-cell contacts (Figure 7C-6D'). These individual neurons can display a variety of morphologies, ranging from dramatically polarized with a clear leading edge to more amorphous and lacking any clear filopodia. First, this observation reinforces the idea that FBMNs migrate as individuals (streams) that make transient physical interactions, and not as a cluster with stable physical adhesions. Second, this suggests transient physical interactions may continually influence the polarity of FBMNs as they migrate.

Pk1b morphant and mutant FBMNs are blocked in r4

To determine whether soma-soma interactions are important for the collective migration mediated rescue of PCP-deficient neurons, I sought to transplant cells from *pk1b* mutant donors into wild-type hosts. The Walsh lab fish facility houses only *pk1b* +/- fish, and therefore to

generate donor embryos for transplanting, I used *pk1b* +/- in-crosses, resulting in 25% of the progeny (*pk1b*-/-) being informative in transplants. Since blastula stage transplants are not 100% effective (for instance, donor cells may differentiate into cells that are not facial neurons), an alternative method for *pk1b* loss-of-function was sought. To generate pk1b-deficient donor embryos, *pk1b* antisense morpholino oligonucleotides (MOs) were utilized, as morpholinos block expression of the target protein and can be utilized as a knockdown. To ensure that I could indeed generate similar phenotypes, the severity of the FBMN migration defect was compared in both *pk1b* mutants and *pk1b* morphants. Both *pk1b* mutants and *pk1b* morphants display FBMNs that fail to migrate out of r4 at 48hpf, when wild-type FBMNs would be fully migrated (Figure 8A-B). *Pk1b* morphants display a full or partial failure to migrate 82.6% of the time (n=98 embryos).

Pk1b morphant FBMNs are rescued by wild-type FBMNs

In order to determine the mode of rescue of *pk1b*-deficient FBMNs, I first sought to confirm the possibility of rescue by wild-type neurons. To test whether placing *pk1b*-deficient neurons adjacent to wild-type FBMNs could rescue of some of the *pk1b*-deficient neurons, I performed blastula stage transplants of cells from *pk1b*-/-;*Tg(isl1:memb-RFP)* donors into wild-type *Tg(isl1:GFP)* hosts. Preliminary transplants imaged at 48 hpf, when wild-type FBMNs have properly migrated into r6/r7, indicate that *pk1b-/-* FBMNs are able to be rescued by wild-type FBMNs (Figure 9A-C). Rescued *pk1b* mutant FBMNs are surrounded by wild-type cells on either side. This initially appears to be consistent with the soma-soma model of migration, suggesting that rescued FBMNs collided with neighboring wild-type FBMNs, inducing CIL behavior that promotes caudal migration. Rescued cells are observed further into r6 and r7,

suggesting that donor *pk1b* mutant cells were able to make many migration promoting collisions with later born wild-type FBMNs. However, without live-image time-lapse imaging of this process in action, it cannot be concluded that this is the method by which FBMNs migrate.

I next sought to determine the mode of rescue of morphant neurons by wild-type neurons by time-lapse live-image of blastula-stage transplants. To accomplish this, I transplanted cells from *pk1b* morphant *Tg(isl1:GFP)* donors to wild-type *Tg(isl1:memb-RFP)* hosts. I observed that morphant FBMNs failed to rescue, though they are making contact with the axon (Figure 9D-F). Some of these FBMNs appear to migrate partially into r5 but are distinct from the fully migrated wild-type population.

Morphant neurons that were not rescued were observed making protrusions towards the pioneer axon (Figure 10). These protrusions are rather long and stable, contact the entire width of the axon (Figure 10A-F). At the final timepoint, these neurons are still present in r4, suggesting that sustained axonal contact is not sufficient for *pk1b* morphant neurons to begin migrating. Other donor FBMNs underwent CIL-typical behavior with wild-type FBMNs (Figure 11). Morphant FBMNs make contact with wild-type FBMNs and migrate away from the site of contact. While no protrusions are visualized, this behavior is still consistent with CIL (Figure 11A-F). This suggests that PCP is not required in the *pk1b*-deficient FBMN for CIL events, which is a necessary condition if CIL is in fact responsible for the rescue of PCP-deficient neurons. Observing CIL behavior between two neurons *in vivo* provides further evidence that migrating cells are making soma-soma contact. However, even though morphant neurons undergo CIL, it is unclear why they are not fully rescued (as seen in Figure 9 D-F and Figure 11G).

Next, I sought to determine why morphant neurons undergoing CIL are not fully rescued. I suggest that while some morphant neurons are born at the right timepoint, allowing wild-type FBMNs to collide with the morphant and encourage its caudal migration, this cell may happen to be jostled out of the way by a CIL collision that results in lateral movement out of the path of other migrating wild-type FBMNs. These wild-type FBMNs then migrate around the morphant FBMN, whose migration stalls.

CIL collisions that do not result in caudal movement were observed (Figure 12). A collision between a *pk1b* morphant neuron results in classic CIL behavior, with both cells withdrawing from the site of collision (Figure 12A-B). This collision promotes caudal directionality in the wild-type cell that continues on in its proper migration path. However, the *pk1b* morphant cell migrates rostrally to collide with another wild-type cell in a more lateral manner. The change in directionality that results from this collision is not fully in the caudal direction (Figure 12 C-D). The *pk1b* morphant FBMN and the adjacent wild-type neuron continue to collide and engage in CIL, with each reversal of direction becoming more and more lateral as development progresses (Figure 12E-F). These continuing collisions, as well as the accumulation of more *pk1b*-morphant FBMNs results in the wild-type FBMN eventually migrating past the morphant neuron (not shown).

This provides further explanation as to why some of these neurons only migrated partially, or there was no rescue. This failure to fully rescue is most likely due to the lack of wild-type neurons rostral to the morphant neuron to induce CIL and migration in the proper caudal direction, and/or due to CIL collisions promoting migration in a lateral direction. Without collisions promoting proper caudal directionality, wild-type cells may bypass morphant neurons and continue their proper migration, leaving the morphant neuron behind.

Here, I have provided evidence that soma-soma contact appears to be the dominant contact in FBMNs migration. Further, evidence presented here indicates that soma-axon contact is not responsible for FBMN migration. Live image time-lapse analysis indicates that the direction of FBMN migration is instead influenced by soma-soma contact that results in CIL.

DISCUSSION

Neuron migration is an essential part of development of the nervous system. Neurons are born in one location and must migrate to their final position in order to properly assemble into circuits. Recent evidence has suggested that neurons engage in collective migration (*Walsh et al. 2011; Davey et al. 2016; Rebman et al., 2016).* As previously discussed, collective migration of neurons has yet to be fully characterized in the developing nervous system. Neuron-neuron interactions are an essential component of the cues that promote the collective behavior of neurons. Previous work using facial branchiomotor neurons (FBMNs) as a model system has uncovered that N-cadherin-mediated neuron-neuron interactions are required for their coordinated movement. To date it has been unclear what the nature of these physical interactions are between neurons that drive the collective behavior of neurons. The studies conducted here have investigated the nature of these neuron-neuron interactions that are important for collective neuron migration.

Previous publications have suggested that the leading axon is necessary for the migration of all following FBMNs (*Wanner & Prince, 2013*). Ablation of the "pioneer" neuron results in stalling of follower neuron migration in 33% of the ablated embryos. On the other hand, in 42% of embryos with ablated pioneer neurons, some follower neurons still migrate into r6, and 25% display a weak phenotype, where most follower neurons migrate into r6 (*Wanner & Prince, 2013*). Further, ablation of only the leading neuron's axon results in 66% of neurons failing to migrate (*Wanner & Prince, 2013*). Ablation of the second follower neuron, rather than the leading neuron, does not result in a stalling of neurons, though a small number of ablated embryos displayed a weak phenotype.

The results presented in this thesis contradict this model. If neurons were solely migrating on the leading axon, I would predict that all neurons would contact the axon in a way that suggests the axon is a necessary substrate for migration, similar to elongated cortical neurons migrating closely on and tightly adhered to radial glia (*Kriegsten et al., 2004*). At early timepoints, neurons do not appear to require axonal contact to migrate out of r4, as suggested by Wanner and Prince, 2013. First, newly born neurons do not adhere immediately to the pioneer axon as a substrate for migration. Further, neurons that do contact the leading axon do not display a morphology that suggests an intimate association with the pioneer axon. FBMNs do not elongate their morphology to wrap around and intimately crawl along the pioneer axon like newly born cortical neurons do on radial glial fibers (*Kriegsten et al., 2004)*. Instead, many FBMNs are observed to be overlying or making transient contact with the axon. The observation that FBMNs overlying the axon is made more prevalent due to confocal projections that collapse image information into one 2-dimensional image. The protrusive activity towards the axon that was observed does not suggest that the leader axon is a necessary substrate. The lack of intimate association with the pioneer axon, both in terms of protrusive activity and morphology of individual migrating FBMNs, suggests that migrating FBMNs do not solely rely on contact with the pioneer axon for migration.

Previous evidence from the Walsh lab (not shown) has also described CIL in the migratory behavior of FBMNs. That is, collision between the soma of adjacent neurons can often lead to collapse of protrusions at the site of contact, followed by migration away from one another. Using mosaic analysis, I showed that an individually labelled FBMN does not make protrusions at sites of soma-soma contact with neighboring FBMNs. Rather, protrusions are made into free space, away from collision sites. This is consistent with traditional CIL examples,

where protrusive activity repolarizes away from the site of contact and directional movement away from the site of contact is observed. FBMNs migrate as individuals, rather than a continuously connected cluster of cells, that make transient contacts with each other. These results provide further evidence that these transient soma-soma contacts between FBMNs are responsible for influencing directional migration. That is, CIL is occurring between migrating FBMNs that make soma-soma contact.

In support of this, quantification of neuron-neuron interactions at early timepoints demonstrates that FBMNs are much more likely to be engaged in soma-soma contact then in soma-axon contact. The presence of soma-soma contact is consistent with the "CIL" model of FBMN collective migration. Taken together, this suggests that soma-soma contact is the physical interaction that drives the collective migration for FBMNs, rather than soma-axon contact. However, neither of these models can be unambiguously confirmed without the addition of live imaging of the rescue of *pk1b* deficient cells. As discussed previously, PCP-deficient cells can be rescued by wild-type cells, typical of collective migration. Therefore, by transplanting PCP-deficient cells into a wild-type background and live-imaging FBMN migration, I can directly observe the physical interactions between wild-type neurons and pk1b-deficient neurons that promote the collective migration-mediated rescue of PCP-deficient cells.

I first ensured that rescue of *pk1b*-deficient was possible by wildtype cells by analyzing the end timepoint of the transplant. Indeed, *pk1b*-deficient cells are able to be rescued by wild-type cells. These rescued cells are flanked by wild-type cells in r6 and r7, consistent with the notion that these wild-type cells influenced through physical interactions the caudal migration of the rescued *pk1b*-deficient neurons.

Analysis of live images of chimeric embryos indicate that the leading axon is not a sufficient substrate for migration. At final timepoints, *pk1b*-deficient neurons that make contact with the wild-type axon do not properly migrate, suggesting that soma-axon interactions do not drive the rescue of pk1b-deficient neurons. If the axon was the necessary substrate for FBMN migration, it is expected that axonal contact is sufficient for the rescue of *pk1b*-deficient neurons. Further, time-lapse imaging shows *pk1b*-deficient neurons making protrusions towards the axon. These protrusions are stable, making extensive, long-term contact with the axon; if FBMNs use the axon as a substrate for migration, this stable axonal contact should allow for the *pk1b*-deficient neuron to initiate migration on the axon. However, *pk1b*-deficient neurons do not adhere to and utilize the axon as a substrate for migration, arguing against the Pioneer model of migration.

Some *pk1b*-deficient cells do appear to partially migrate, but stall in r4 or r5. If the "Pioneer" model was the proper mode of FBMN migration, any morphant neuron contacting the axon should rescue fully; however, this is not what occurs. Only the "CIL" model provides a reasonable explanation for partially rescued morphant neurons that stall in r5 or earlier. This could reflect several collisions between wild-type neurons and a *pk1b* morphant neuron that drive a small amount of caudal movement. Time-lapse imaging indicates that wild-type cells collide with *pk1b* morphant cells. CIL behavior between colliding morphant and wild-type FBMNs has been observed, where a morphant cell and wild-type cell contact each other and the morphant neuron migrates away from the site of contact. This suggests that PCP is not required (at least in one cell) for CIL events in FBMNs, which differs from the CIL-mediated collective migration of neural crest cells. While both FBMNs and NCCs require N-cadherin for CIL behavior, NCCs further require PCP for CIL behavior (*Rebman et al., 2016; Becker et al., 2013.,*

Carmona-Fontaine et al., 2008). Inhibition of PCP signaling in NCCs results in protrusions failing to collapse after a collision (*Carmona-Fontaine et al., 2008)*. In migrating FBMNs, collisions between wild-type and PCP-deficient cells result in proper CIL behavior, differentiating the collective migration of FBMNs from that of NCCs. These collisions do not always promote caudal movement and instead jostle the *pk1b* morphant cell out of the path of other wild-type cells, and then the wild-type neuron by-passes the stalled *pk1b*-deficient neuron (Figure 13). In the absence of further collisions with wild-type neurons, the *pk1b*-deficient neurons are no longer rescued by CIL-causing soma-soma interactions. This is one reason why I had difficulty imaging the CIL-mediated rescue of *pk1b*-deficient cells, as expected.

A second potential explanation for why time-lapse imaging the rescue of *pk1b*-deficient neurons proved to be difficult may stem from differences in transplant methodology. Gastrula-stage transplants allow for more specific targeting of donor cells to the prospective ventral hindbrain progenitor domains where FBMNs will arise. Gastrula-stage transplants have previously shown that PCP-deficient FBMNs are rescued by wild-type FBMNs; however, only about 50% of PCP-deficient FBMNs are rescued (*Rohrschneider et al., 2007; Walsh et al., 2011; Davey et al., 2016*). Blastula-stage transplants, on the other hand, have not been used to show PCP-deficient FBMN rescue in the same manner until now and have proven difficult in targeting the proper cell population and timepoint. Often, transplanted donor cells differentiate into FBMNs at too late of a timepoint to be properly rescued by host wild-type cells that have already progressed in their migration. For instance, an ideal scenario would have donor *pk1b*-deficient neurons born first, followed by wild-type host neurons. The later born wild-type neurons would then collide with the earlier-born PCP-deficient neuron, promoting CIL-induced protrusion formation on the caudal side of the *pk1b*-deficient neuron, leading to caudal movement.

However, in blastula-stage transplants, the *pk1b*-deficient neurons often arose as late-born FBMNs and failed to engage in sufficient numbers of collisions with wild-type host neurons. Similarly, blastula-stage transplants often only give rise to only two or three donor FBMNs, rarely yielding the high number of donor FBMNs seen in gastrula-stage transplants (*Walsh et al., 2011; Davey et al., 2016*).

While I cannot yet unambiguously determine which model is most likely responsible for the collective migration of FBMNs, the evidence presented here suggests that axon-soma contact is neither necessary nor sufficient for FBMN migration. Fixed tissue imaging shows that FBMNs do not contact the axon at early timepoints, and *pk1b*-deficient FBMNs that contact the axon do not rescue and properly migrate. Observations of soma-soma contact and loss of protrusions at sites of soma-soma contact are consistent with the CIL-model of neuron migration. The live imaging done here has provided evidence for CIL occurring between migrating neurons and influencing the direction of migration. Future live imaging of rescue of *pk1b*-deficient neurons will resolve unambiguously that soma-soma contact, or the "CIL" model, is solely responsible for the collective migration of FBMNs.

Understanding the cellular nature of the transient contacts that mediate collective migration of migrating neurons could lead to further insight into resolving developmental defects. Neuron migration defects can result in severe developmental disorders, as previously discussed. Having a full understanding of the developmental context of neuron migration could lead to future medical breakthroughs that allow rectification of migration defects.

Future directions

Blastula-stage transplants, while often successful, do not allow for specific targeting of FBMNs. Gastrula-stage transplants could be considered as an alternative transplantation method. Similarly, transient transgenesis through the introduction of a wild-type *pk1b* plasmid into *pk1b-/-* could result in a different method of visualizing rescue. Mutant cells could be rescued by reintroduction of full-length Pk1b protein and could therefore behave like wild-type cells and rescue neighboring mutant cells. Further, there are other ways to visualize CIL in migrating FBMNs. Injecting constructs that allow us to visualize cell machinery as it re-orients *in vivo* after a collision event would provide further evidence that CIL is occurring in migrating FBMNs.

F-actin polymerizes at the leading edge of a migrating cell, stabilizing exploratory protrusions. When cells undergoing CIL collide, protrusions are collapsed at the site of collision and actin repolymerizes at the opposite pole of the cell, enabling protrusions to be made in the opposite direction from the site of contact (*Stramer & Mayor, 2016*). Injecting a fluorophore tagged Lifeact into either the *Tg(isl1:GFP-CaaX)* or *Tg(isl1:mRFP)* lines would allow visualization of F-actin dynamics in FBMNs *in vivo*. If CIL is occurring in FBMNs during migration, a collapse of F-actin at the site of collision and a repolarization to the opposite pole of the migrating cell is expected. This would result in directional migration in the opposite direction of the collision in FBMNs and provide further evidence that CIL is occurring in FBMNs.

Illustrating repolarization of the cell also could be done by visualizing centrosome location. Centrosomes are an essential cytoskeletal organization center in migrating cells, regulating microtubule organization (*Kuijpers et al., 2011*). Centrosomes in migrating cells are localized ahead of the nucleus, from which microtubules extend out towards the leading edge of the migrating cell. Improper localization of centrosomes in FBMNs has resulted in aberrant

migration in FBMNs (*Grant and Moens, 2010).* This suggests that proper centrosome localization is important for regulating directional migration of FBMNs, and therefore may play a role in reorienting cells after CIL collision events. Visualization of the location of the centrosome by injection of a fluorophore tagged centrin, a protein that localizes to the centrosome, would also allow us to visualize movement of the centrosome after a CIL collision event, as I could watch the centrosome reorient opposite of the site of collision. This reorientation would lead to the directional change in movement that is typical of CIL. This would provide further evidence for the "CIL" model of collective FBMN migration.

The "Pioneer" model and "CIL" model are not mutually exclusive; while most FBMNs are not contacting the axon, it is possible that some migrating cells do require axonal contact to properly migrate. This is challenging to visualize in maximum projections of confocal images, as cells that may not be close in 3D space appear to be overlaying each other in a 2D projection. To further establish that FBMNs are migrating independent of axon, using a microtome to make micron-thick sections of *Tg(isl1:GFP-CaaX)* embryos embedded in JB4 resin that preserves fluorescence would allow for high resolution imaging to visualize whether FBMN axons are indeed making contact with leader axons.

FIGURES

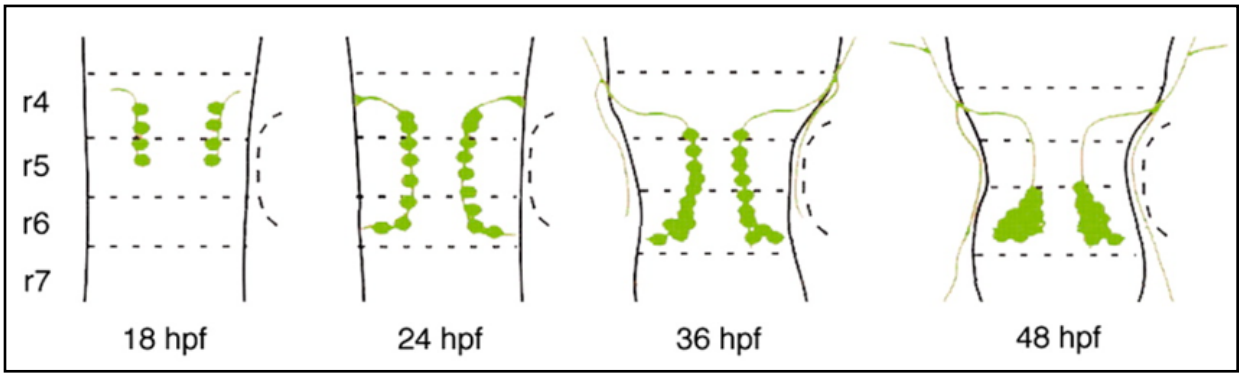

Figure 1: Facial branchiomotor neuron migration is highly conserved. FBMNs (in green) are born around 16hpf in r4 of the developing hindbrain. They migrate posteriorly until they reach r6 at 48hpf.

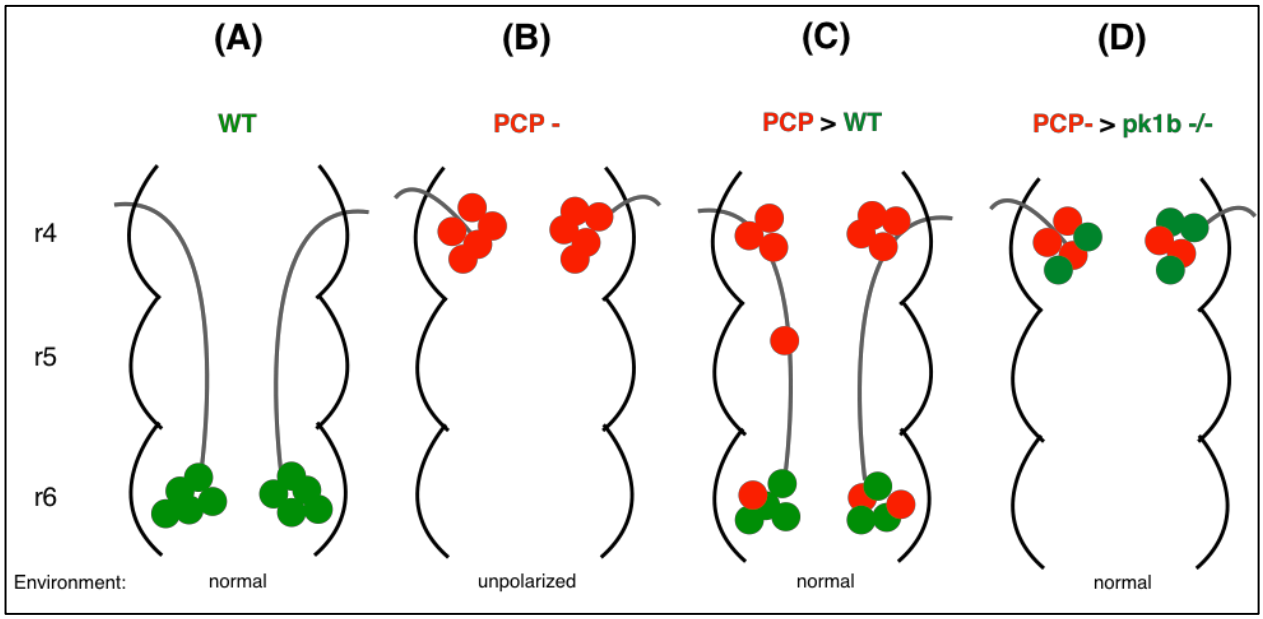

Figure 2. **Cell transplantation experiments to show cell autonomy**. Some PCP mutant neurons migrate when transplanted into WT hosts. PCP mutant neurons do not migrate though pk1b-deficient environment due to lack of collective migration.

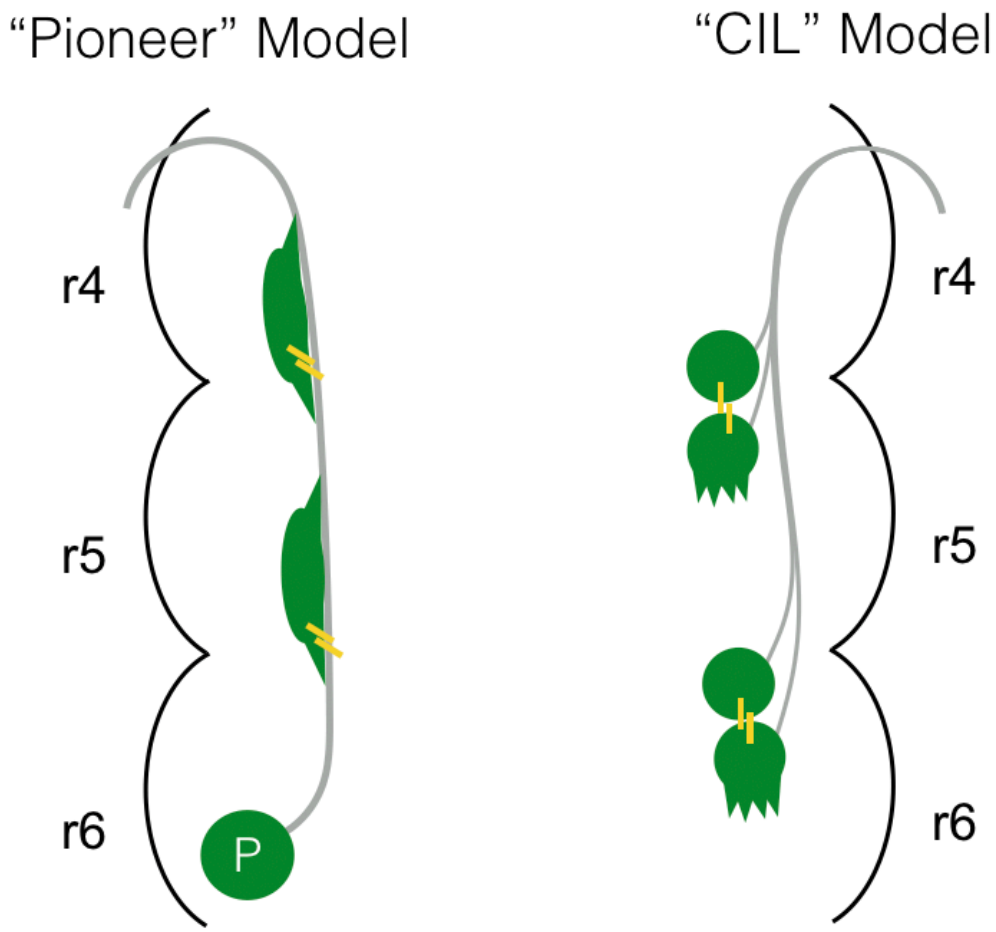

Figure 3. Two potential models for the cellular mechanisms of collective FBMN migration. FBMNs (in green) migrate intimately on the axon of the leader cell (Pioneer model) or migrate by engaging in cell-cell contact that leads to repolarization and caudal movement (CIL model). Both the pioneer model and CIL model are mediated by N-cadherin (yellow) dependent cell-cell contact.

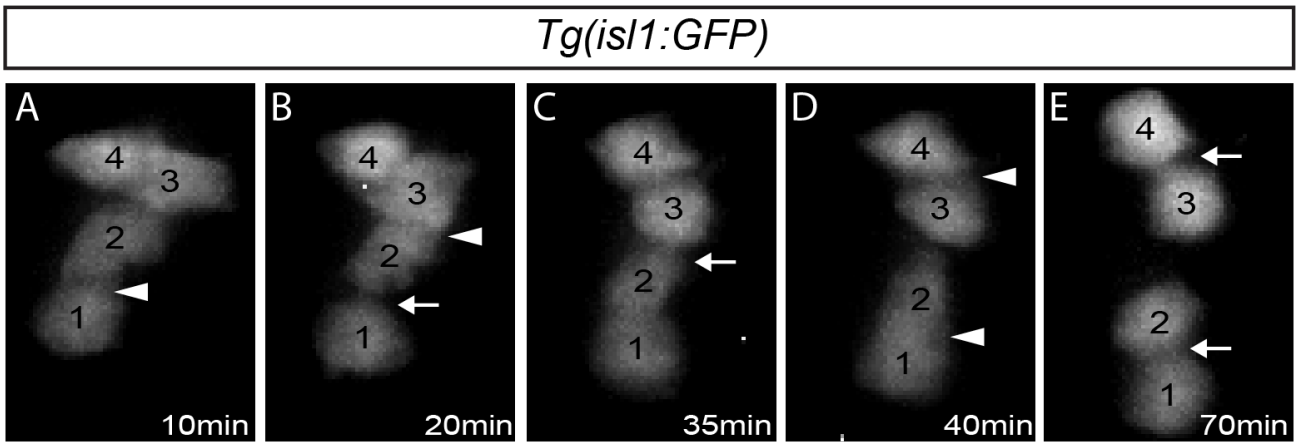

Figure 4. Wild-type FBMNs undergo CIL *in vivo*. Collisions between migrating wild-type cells result in a repolarization and colliding cells withdrawing from sites of contact. Cells that undergo collisions begin to migrate in the opposite direction from the site of collision. (A-E) Live confocal imaging of wild-type cells. (Arrowheads denote sites of contact; arrows indicate sites of contraction and cells migrating away from the site of contact.)

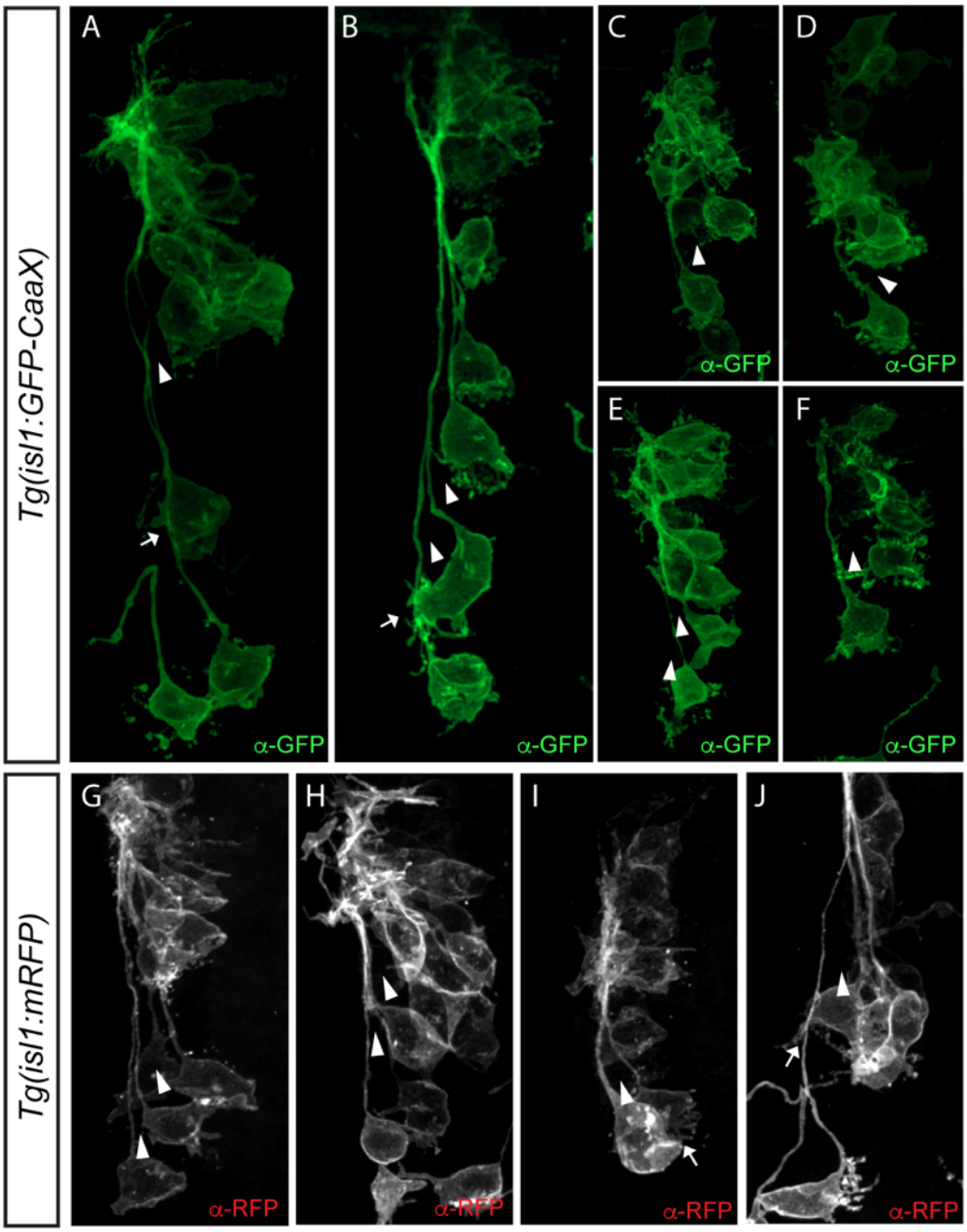

Figure 5. Early born FBMNs do not make contact with the leader axon. FBMNs visualized contacting the axon made transient contacts and did not display an intimate association (A,B, J; arrows indicate soma-axon contact; arrowheads indicate space between leader axon and migrating FBMNs). FBMNs appear to migrate as clusters, contacting other FBMNs and not the axon (C-G; arrowheads indicate space between leader axon and migrating FBMNs). While axon fasciculation between two FBMNs was observed, following cells were contacting other FBMNs rather than the axon (H, I; arrowheads indicate sites of fasciculation; arrow indicates soma-soma contact). (A-F) Fixed tissue confocal imaging of anti-GFP immunofluorescence in *Tg(isl1:GFP-CaaX)* embryos. (G-H) Fixed tissue confocal imaging of anti-RFP immunofluorescence in *Tg(isl1:mRFP)* embryos.

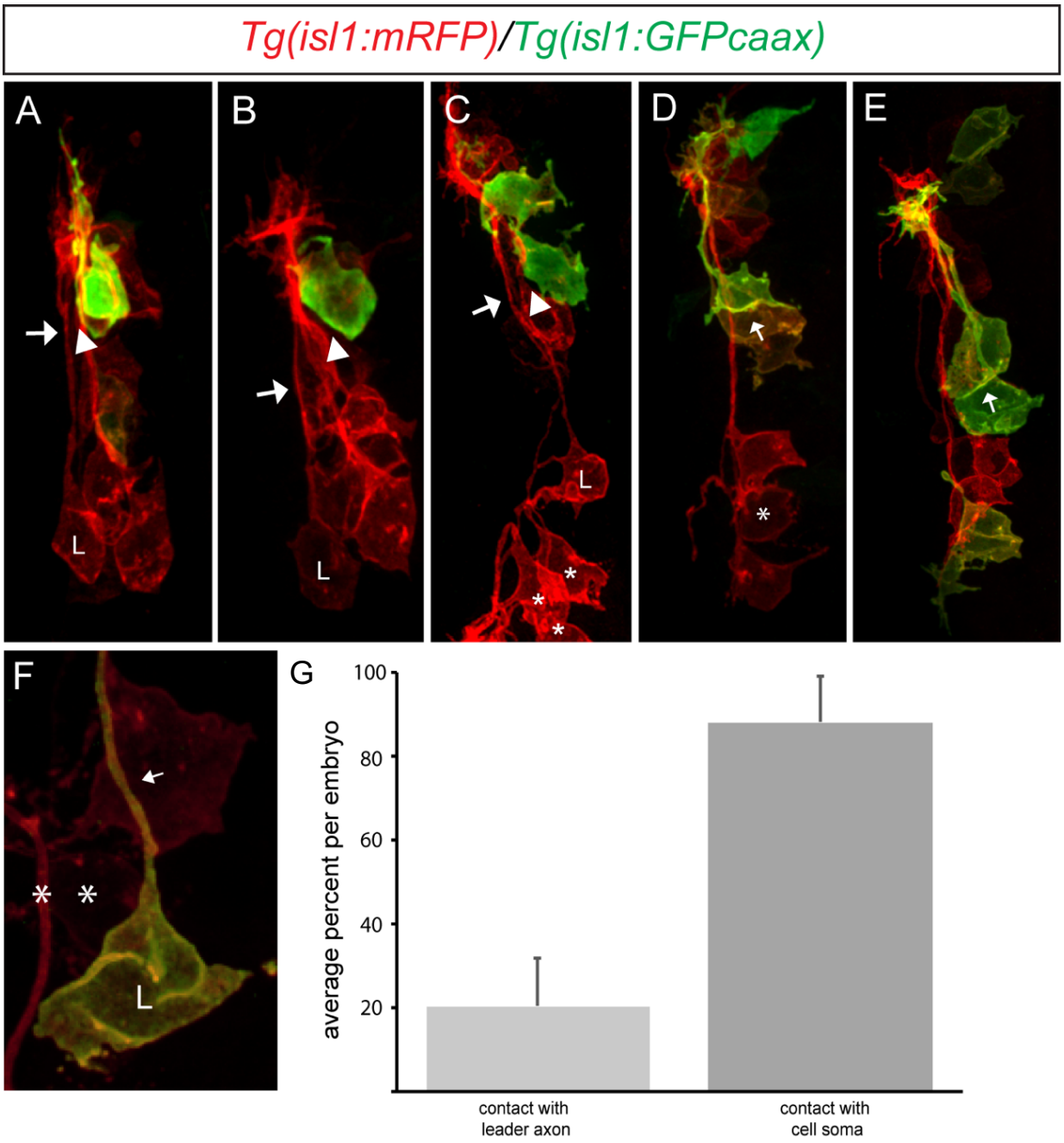

Figure 6. FBMNs are more likely to make soma-soma contact than soma-axon contact. FBMNs migrating out of r4 do not adhere to the leader axon as they begin their caudal migration (A-C). Migrating FBMNs make soma-soma contacts coupled with a lack of protrusions at sites of contact (D, E). Most FBMNs making axonal contact are overlaying the leader axon, rather than displaying elongated morphology expected of FBMNs migrating on an axon (F; *denotes r6 derived cells; arrow indicates axon-soma contact). 20% of early born migrating FBMNs make contact with the leader axon, while 88% make soma-soma contact (n= 26 embryos, 339 FBMNs; $p < .0001$) (G). (A-F) Fixed tissue confocal imaging of anti-RFP and anti-GFP immunofluorescence in mosaic *Tg(isl1:mRFP)* embryos injected with an *isl1:GFP-CaaX* construct.

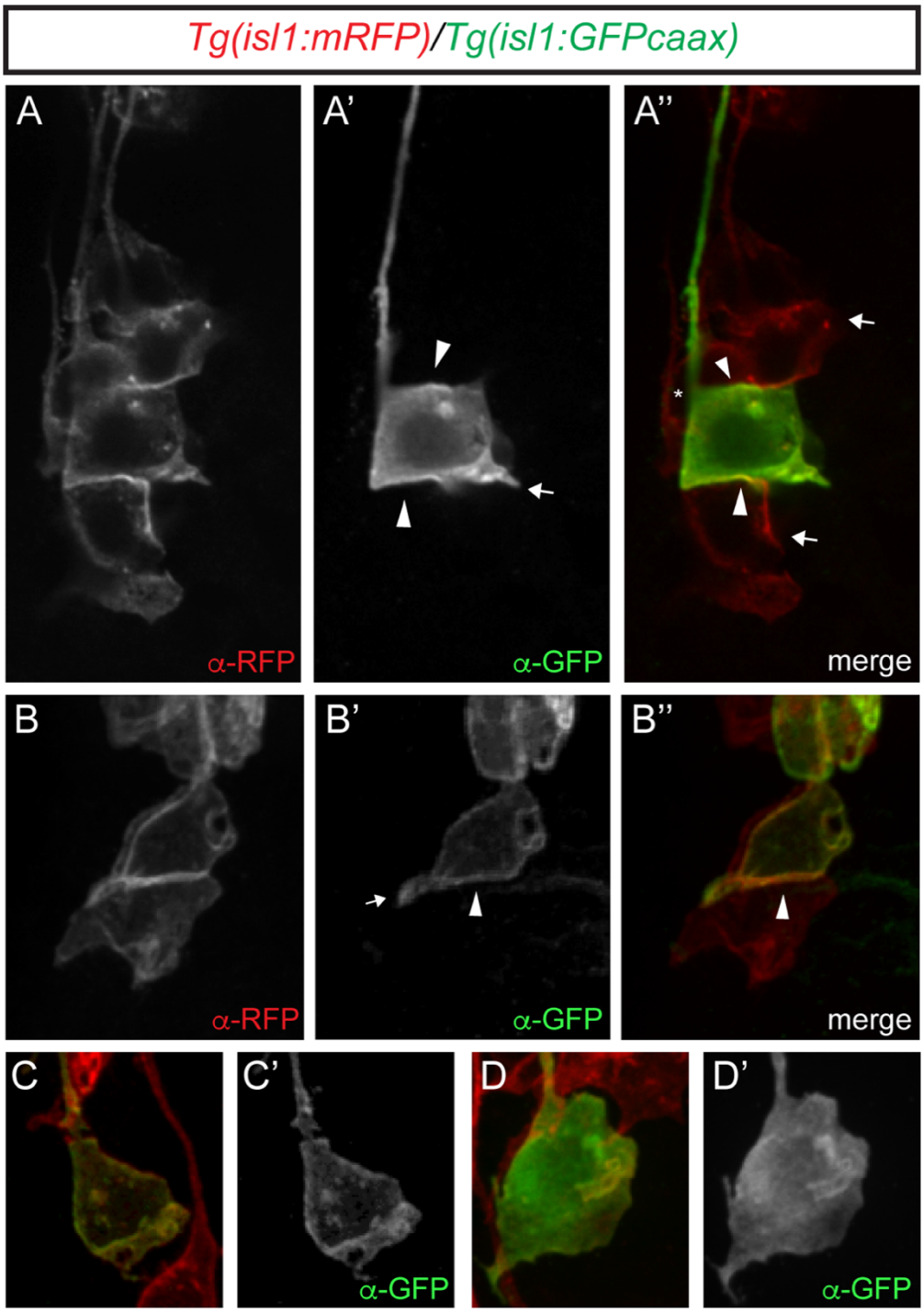

Figure 7. FBMNs migrate as individuals and display CIL typical behavior. Mosaic embryos allow for visualization of protrusion collapse at sites of contact. FBMNs making soma-soma contact display protrusive activity away from the contact site (A-B''). Rather than remaining tethered to each other, FBMNs migrate as individuals, and may nor may not display protrusive activity in the absence of a collision event (C-D''). (A-D') Fixed tissue confocal imaging of anti-RFP and anti-GFP immunofluorescence in mosaic *Tg(isl1:mRFP)* embryos injected with an *isl1:GFP-CaaX* construct.

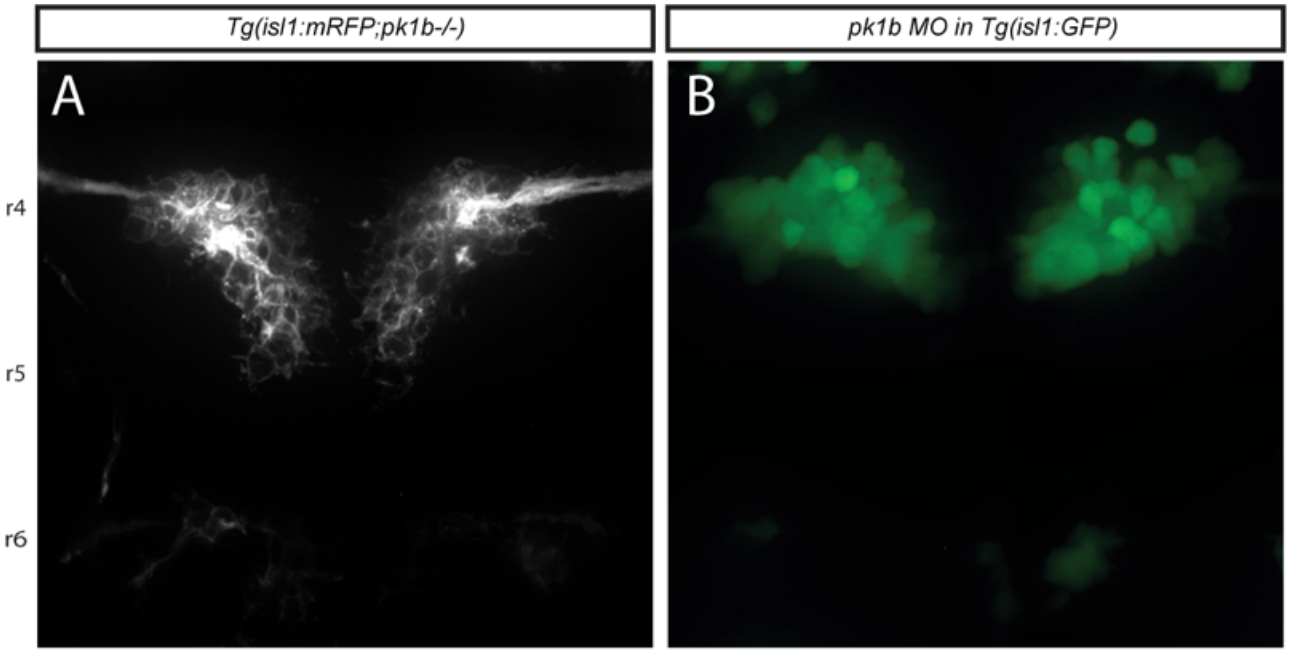

Figure 8. Prickle1b-deficient FBMNs are blocked in r4. *Pk1b-/-* FBMNs fail to migrate out of r4 (A). *Pk1b* morphant neurons similarly fail to migrate out of r4 (B). 82.6% of morphant embryos display a strong phenotype (failure of all neurons to migrate out of r4; n=98 embryos).

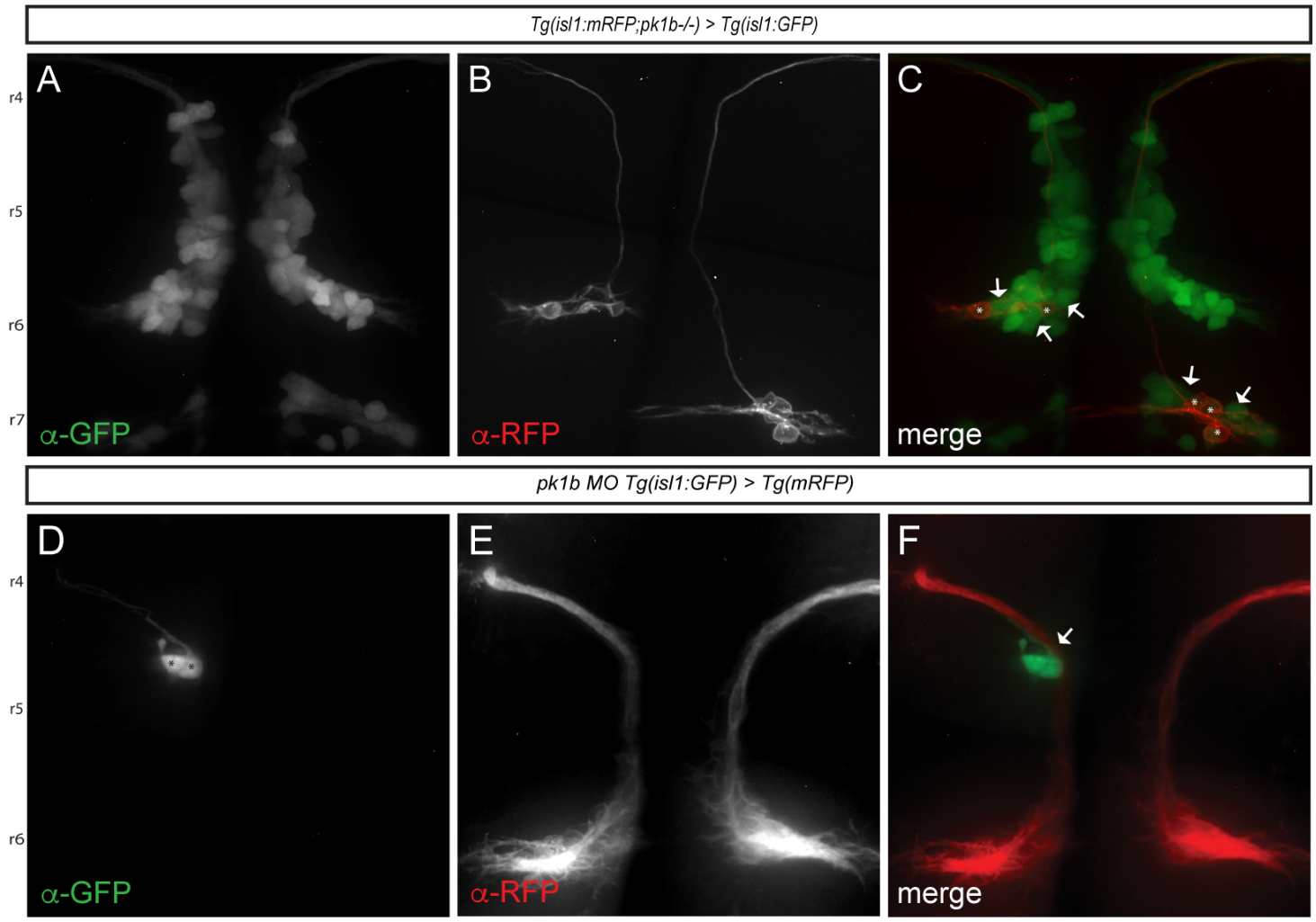

Figure 9. Prickle1b-deficient neurons can be rescued by wild-type cells. *Pk1b*-deficient neurons may be rescued by wild-type neurons when transplanted into a wild-type host (A-C). Note that rescued neurons are usually surrounded by neurons on either side (* denotes rescued cells; arrows denote surrounding wild-type cells). Non-rescued *pk1b*-deficient neurons appear to make contact with the axon and may have partially migrated into r5, but do not migrate into r6 (D-F). Sustained axonal contact does not appear to be sufficient enough to rescue *pk1b*-deficient neurons (* denotes two morphant cells; arrow indicates site of soma-axon contact). (A-C) Live confocal imaging of blastula stage transplants of *Tg(isl1:mRFP;pk1b-/-)* into *Tg(isl1:GFP)* at 48hpf. (D-F) Live confocal imaging of blastula stage transplants of pk1b morpholino in *Tg(isl1:GFP)* into *Tg(isl1:mRFP)* at 48hpf.

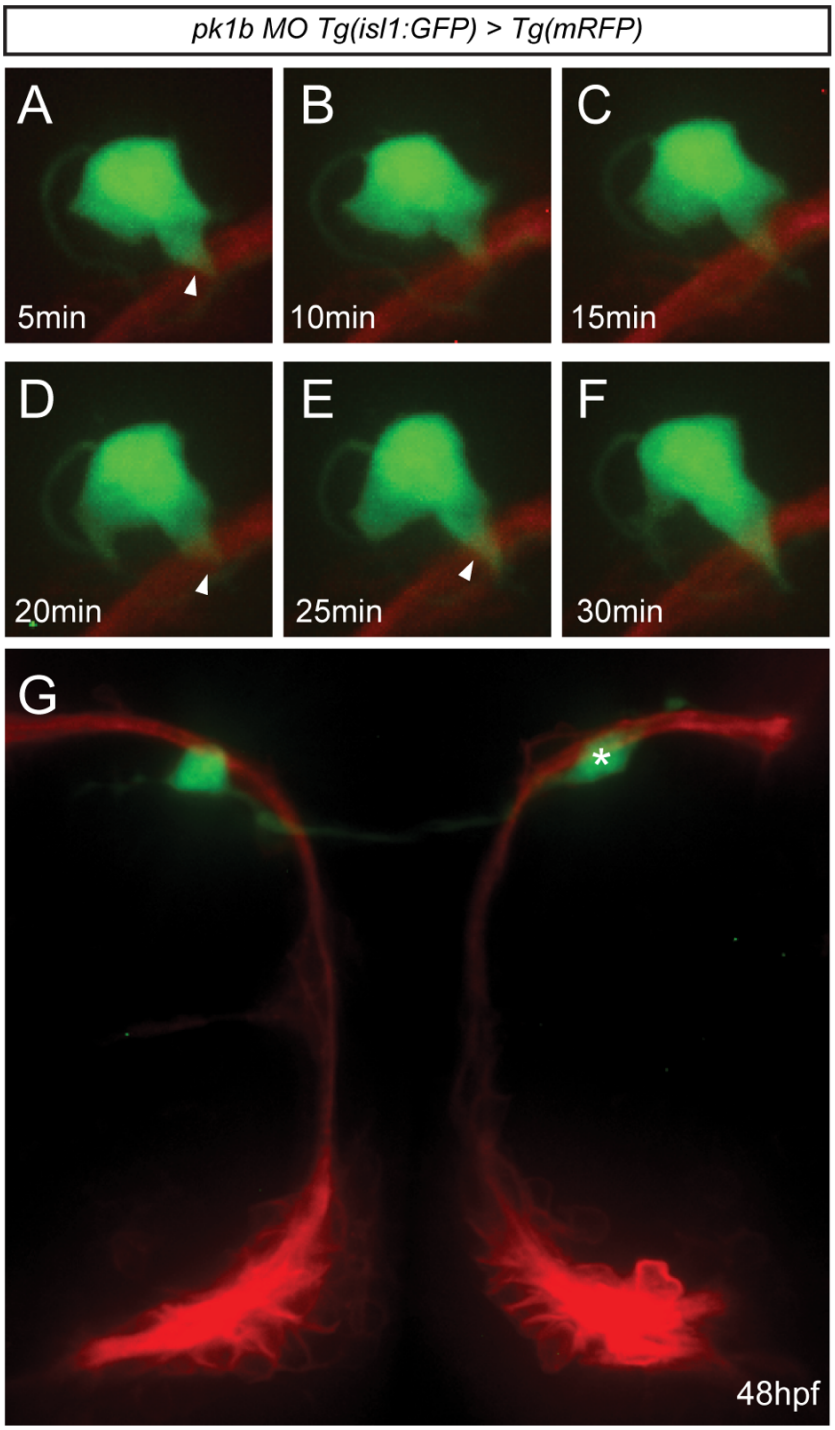

Figure 10. Soma-axon contact is not sufficient to rescue pk1b-deficient FBMNs. Pk1b morphant neurons display stable protrusive activity towards the axon (**A-F**). Protrusions contact the axon (A-D) or even continue past the axon (E-**F**). Axonal contact does not result in these neurons properly migrating into r6, as they remain blocked in r4 (* denotes FBMN in A-F) (**G**). (A-F) Live confocal imaging of blastula stage transplants of pk1b morpholino in *Tg(isl1:GFP)* into *Tg(isl1:mRFP)* at 24hpf. (G) Live confocal imaging of blastula stage transplants of pk1b morpholino in *Tg(isl1:GFP)* into *Tg(isl1:mRFP)* at 48hpf.

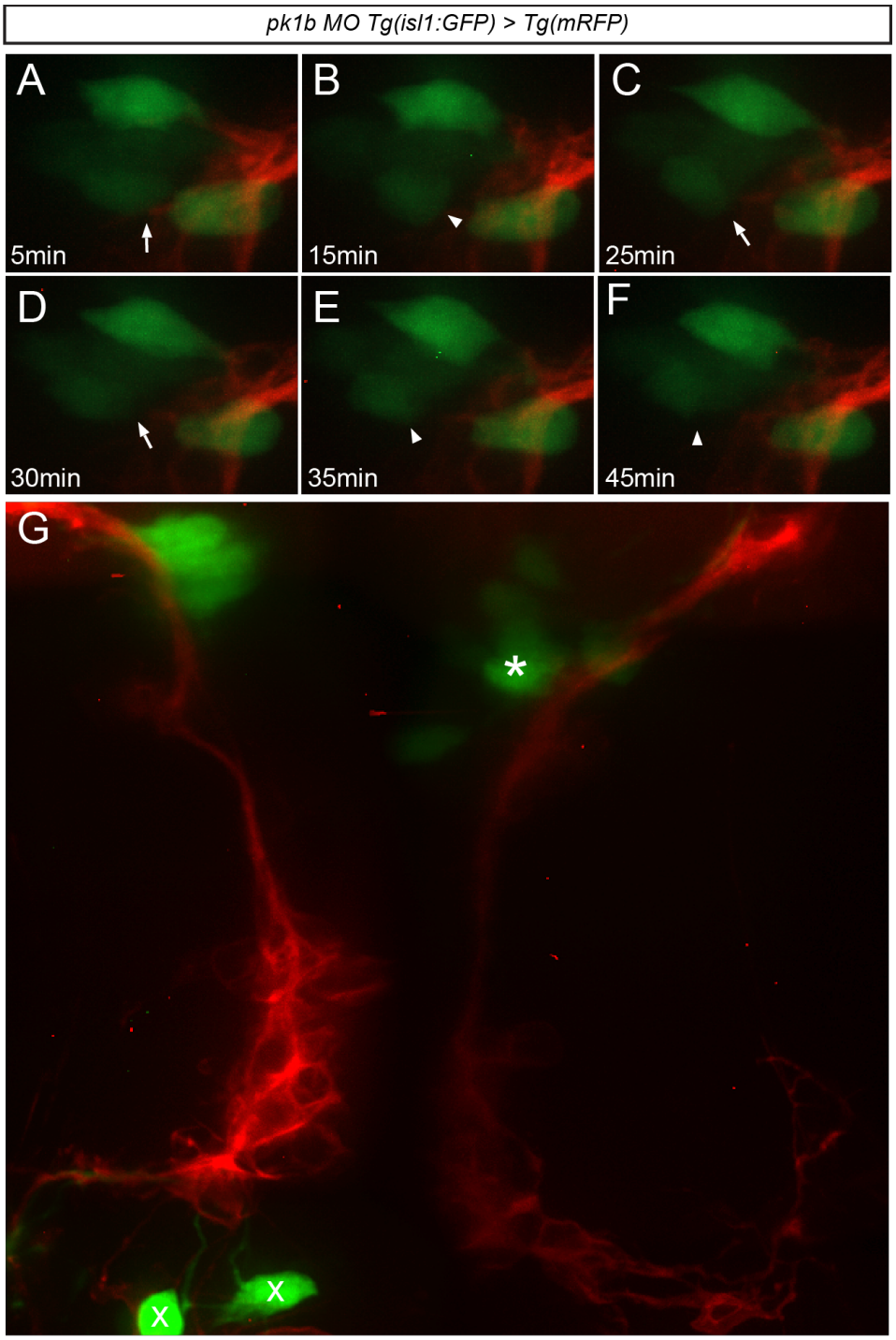

Figure 11. CIL events occur between pk1b morphant FBMNs and migrating wild-type FBMNs. Pk1b morphant neurons make contact with wild-type neurons (arrows) followed by a directional change in movement (arrowheads) (**A-F**). Morphant FBMNs still do not properly migrate (**G**). (A-F) Live confocal imaging of blastula stage transplants of pk1b morpholino in *Tg(isl1:GFP)* into *Tg(isl1:mRFP)* at 24hpf. (G) Live confocal imaging of blastula stage transplants of pk1b morpholino in *Tg(isl1:GFP)* into *Tg(isl1:mRFP)* at 48hpf. ("x" denotes r6 derived cells.)

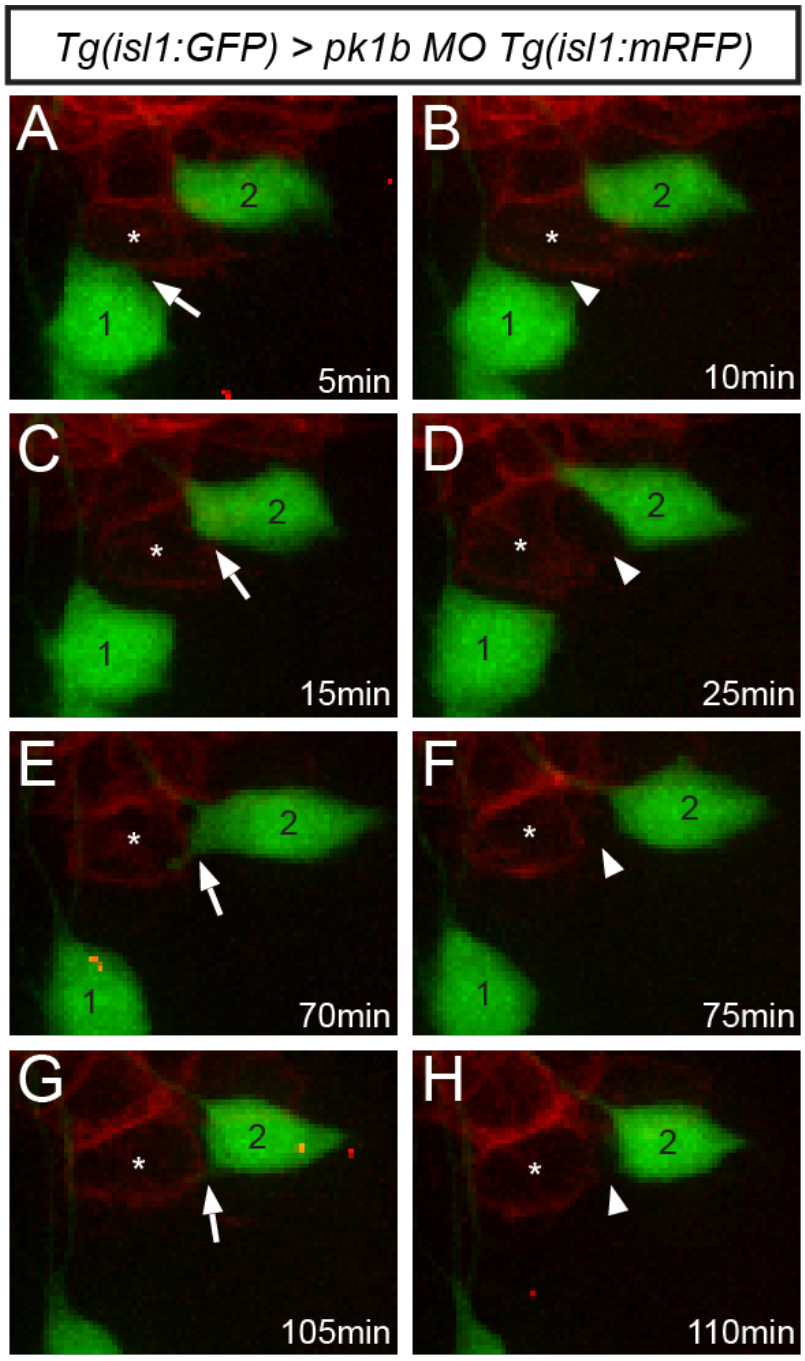

Figure 12. CIL collisions between *pk1b* morphant FBMNs and wild-type FBMNs do not always promote caudal migration. Wild-type FBMNs collide with a *pk1b* morphant neuron and display a reversal of direction (A-H). Collisions are denoted with arrows; directional changes are denoted with arrowheads. Some collisions result in further caudal movement as seen between the pk1b morphant cell (*) and a wild-type cell (1), which will continue its proper migration (A-B). Other collisions between the morphant and a different wild-type cell (2) jostle the wild-type FBMN. This cell is unable to promote caudal migration; rather, CIL events between the *pk1b* morphant and (2) occur repeatedly and result in lateral movement. (A-H) Live confocal imaging of blastula stage transplants of pk1b morpholino in *Tg(isl1:mRFP)* into *Tg(isl1:GFP)* at 24hpf.

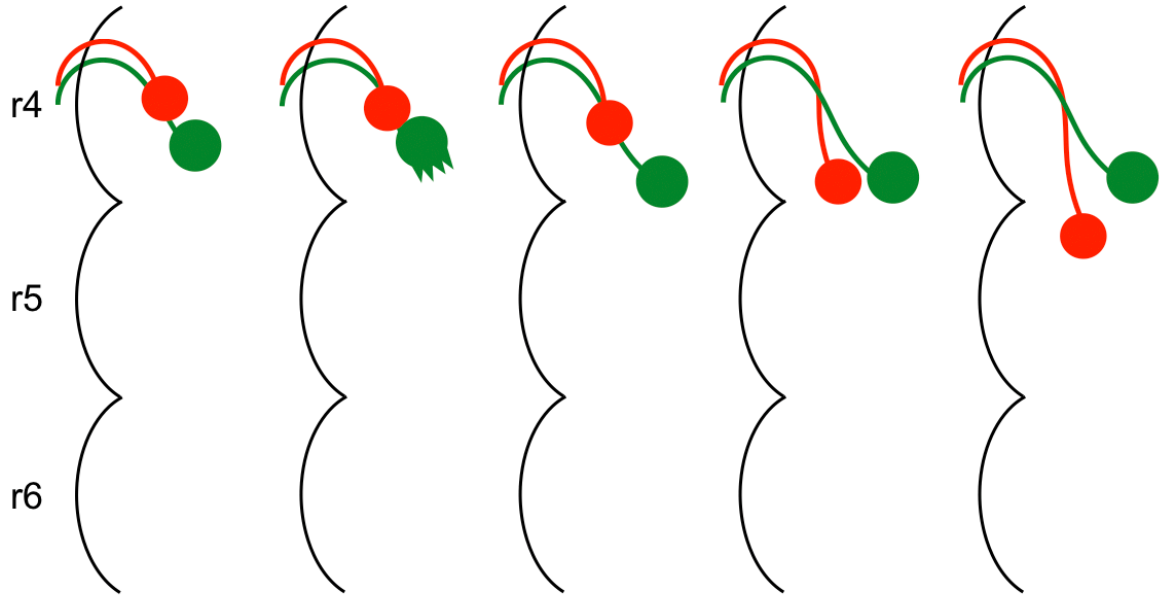

Figure 13. *pk1b* morphant FBMNs engage in CIL with wild-type FBMNs that results in wild-FBMNs cells migrating around them. A wild-type FBMN (in red) collides on an angle with a morphant FBMN (green). This collision results in the *pk1b* morphant cell migrating laterally, and the morphant cell does not engage in further collisions with wild-type FBMNs. The wild-type neuron migrates normally, bypassing the *pk1b* morphant neuron.

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VITA

Rebecca Damayanthi Vareed was born on October 20th, 1995 in Nagercoil, Tamil Nadu to Zinda Guldager. She graduated *magna cum laude* from Virginia Commonwealth University in 2017 with a Bachelor of Science in biology. After receiving her Master of Science in Biology from Virginia Commonwealth University, she will pursue a Ph. D. in Neuroscience at the University of Colorado Anschutz Medical Campus.