The Role of Planar Cell Polarity Signaling in Neuronal Migration

Crystal Faye Davey Hicks

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Reading Committee:

Cecilia Moens, Chair

David Kimelman

Valeri Vasioukhin

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Abstract

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Crystal Faye Davey Hicks

Chair of the Supervisory Committee:

Cecilia B. Moens, PhD

Division of Basic Sciences, Fred Hutchinson Cancer Research Center

The planar cell polarity (PCP) pathway is a cell-contact mediated mechanism for transmitting polarity information between neighboring cells. PCP “core components” (Vangl, Fz, Pk, Dvl, and Celsr) are essential for a number of cell migratory events including the posterior migration of facial branchiomotor neurons (FBMNs) in the plane of the hindbrain neuroepithelium in zebrafish and mice. While the mechanism by which PCP signaling polarizes static epithelial cells is well understood, how PCP signaling controls highly dynamic processes like neuronal migration remains an important outstanding question given that PCP components have been implicated in a range of directed cell movements, particularly during vertebrate development. Here, I present evidence that PCP signaling is required both within FBMNs and in the hindbrain rhombomere 4 environment at the time when they initiate their migration. Correspondingly, I demonstrate planar polarized localization of PCP core components Vangl2 and Fzd3a in the

hindbrain neuroepithelium, and transient localization of Vangl2 at the tips of retracting FBMN filopodia. Using high-resolution timelapse imaging of FBMNs in genetic chimeras I uncovered opposing cell-autonomous and non-cell-autonomous functions for Fzd3a and Vangl2 in regulating FBMN protrusive activity. Within FBMNs, Fzd3a is required to stabilize filopodia while Vangl2 has an antagonistic, destabilizing role. However, in the migratory environment Fzd3a acts to destabilize FBMN filopodia while Vangl2 has a stabilizing role. Together, my findings suggest a model in which PCP signaling between the planar polarized neuroepithelial environment and FBMNs directs migration by the selective stabilization of FBMN filopodia.

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Chapter 1: Introduction

Planar cell polarity (PCP) is a feature of many animal tissues. This type of polarity is most obvious in cells that are organized into epithelial sheets, where cells exhibit two types of polarization, apicobasal polarity and planar polarity, which refers to cell polarization in the plane orthogonal to the apicobasal axis. While apico-basal polarity mediates, and limits, the communication of cells and tissues with the outside world, the fundamental function of PCP is the contact-dependent communication of directional information between adjacent cells and thereby across entire tissues. The outcomes of PCP are diverse, ranging from the orientation of single cells within an epithelium to the orientation of multicellular epithelial structures such as the mammalian hair follicle or the fly ommatidium, to the oriented movements of motile cells in the developing embryo. However all of these processes are united by their use of a core group of membrane associated proteins that influence the organization of the cytoskeleton to bring about these diverse outcomes in different cell types. The principles of PCP were elucidated in the fly wing, where planar polarity is easily discerned by the presence of a single actin-rich hair, the trichome, on the distal side of the apical surface of each epithelial cell, and this work forms the conceptual framework for understanding PCP-dependent processes. A number of recent reviews have discussed the establishment and function of PCP in epithelial cells (for review see,Goodrich and Strutt, 2011; Gray et al., 2011; Carvajal-Gonzalez and Mlodzik, 2014; Devenport, 2014). Here I will focus on the role of PCP in oriented cell movements, beginning with the coordinated, coherent movements of similar cells within a tissue. However, PCP components have recently been implicated in a number of directional cell migrations *in vivo* that involve interactions between two or more different kinds of cells, and I will consider how PCP signaling between different cell types may control long-range directional cell migrations *in vivo*. In this introduction

I aim to provide insights into the mechanisms by which PCP signaling can control a variety of cell migratory events and I will focus on mechanism as opposed to solely the involvement of PCP.

The Basics of the PCP Pathway: Lessons from *Drosophila*

Two main pathways function in the establishment of planar polarity: the "core" planar cell polarity (PCP) pathway and the Fat/Dachsous (Fat/Ds) pathway (for review, see Matis and Axelrod, 2013). Here I consider exclusively the core PCP pathway, as the role of the Fat/Ds pathway in directional cell movements is less well established. The core PCP pathway is comprised of six members, identified on the basis of having similar mutant phenotypes. Early in wing development, these proteins are symmetrically localized around the apical cell membrane and as development proceeds they become asymmetrically localized in the plane of the epithelium (Tree et al., 2002; Das et al., 2004). The molecular asymmetry of these protein complexes precedes morphological asymmetry and loss of any of these core proteins leads to loss of both molecular and morphological asymmetries. The transmembrane protein Frizzled (Fz) is confined to the distal cell junctions along with the cytosolic proteins Disheveled (Dsh) and Diego (Dgo), while the transmembrane protein Van Gogh/Strabismus (Vang/Stbm) and the cytosolic protein Prickle (Pk) are proximally localized (Fig. 1). The atypical cadherin Flamingo (Fmi) is localized to both proximal and distal membranes. Prior to becoming polarized in the plane of the epithelium, PCP proteins are recruited to the apical membrane (Wu et al., 2004). Fz physically interacts with Dsh and recruits Dsh to the apical membrane (Wong et al., 2003; Wu et al., 2004). Similarly, Vang physically interacts with both Pk and Dsh and Vang is required to recruit Pk and Dsh to the membrane (Bastock et al., 2003; Jenny et al., 2003). The asymmetric

localization of the two PCP complexes results both from intracellular antagonistic interactions between core components (Tree et al., 2002; Jenny et al., 2005) and positive intercellular interactions with neighboring cells (Chen et al., 2008; Wu and Mlodzik, 2008) that both stabilize an initial asymmetry and transmit it across the epithelium. This important non-autonomous effect is apparent in genetic mosaics where clones of cells that lack Fz reverse the polarity of distal wild type neighbors so that their trichomes point towards the mutant clone, and clones of Vang mutant cells reverse the polarity of proximal neighbors so that their trichomes point away from the mutant clone (Vinson and Adler, 1987; Taylor et al., 1998; Adler et al., 2000). This suggests that mutant cells and their wild type neighbors can only set up junctional contacts of a certain polarity and highlights the importance of cell-cell contacts in propagating planar cell polarity.

The core PCP pathway is sometimes referred to as the "non-canonical" Wnt pathway, because it involves Fz-family receptors and Disheveled homologues in a β-catenin-independent signaling pathway (for review, see Strutt, 2003; Veeman et al., 2003a). Given that Fz is a well-known Wnt receptor that activates Dsh upon binding to Wnt, it has been proposed that a Wnt gradient could act as a global polarity cue to establish planar polarity across a tissue (Adler et al., 1997). The role of Wnts in Drosophila planar polarity has been debated (Lawrence et al., 2002; Chen et al., 2008; Trichas et al., 2012), but recently a role for Wnts in establishing wing PCP via modulation of the intercellular Fz-Vang interaction has been demonstrated (Wu et al., 2013). As discussed below, Wnt ligands are required for some PCP processes in vertebrates, however, a requirement for a global Wnt gradient in establishment of planar polarity has been demonstrated in only a few cases (Qian et al., 2007; Gao et al., 2011; Andre et al., 2012).

Several "effectors", factors that act downstream of the core PCP pathway to mediate its effects on cell morphology and behavior have been identified. Fuzzy (Fy) (Collier and Gubb,

1997), Inturned (In) (Park et al., 1996) and Fritz (Frtz) (Collier et al., 2005) represent a group of proteins that seem to act solely as PCP effectors. These proteins colocalize with Vang and Pk at the proximal side of the cell and regulate the localization of the downstream effector Multiple Wing Hairs (Mwh), a formin-homology domain containing protein that antagonizes actin polymerization, restricting hair formation to the distal side of the cell (Strutt and Warrington, 2008; Yan et al., 2008). Other proteins also considered, though disputed, to be core PCP effectors in *Drosophila* include the p21 GTPase RhoA and its effector *Drosophila* Rho associated kinase (Drok), both of which modulate the cytoskeleton via regulation actin polymerization (Strutt et al., 1997; Fanto et al., 2000; Winter et al., 2001; Chung et al., 2007). Additionally, the c-Jun N-terminal kinase (JNK) pathway, which in addition to its role in regulating transcription is purposed to mediate cell motility (for review, see Xia and Karin, 2004), is activated downstream of Fz and Dsh to affect planar polarity in the *Drosophila* eye (Boutros et al., 1998; Weber et al., 2000).

Planar polarity is a well-defined cell property in the epithelial cells of the *Drosophila* wing and eye, which has permitted the characterization of the proteins and signaling events that mediate its establishment. However, as we discuss below, study of the core PCP proteins in other systems have demonstrated roles for these proteins in cells that are not classically epithelial, and in tissues that do not display obvious planar organization. Herein, I will refer inclusively to processes involving PCP proteins as “PCP signaling” events or “PCP processes”. However, as others have noted, a process involving one or more of these proteins does not necessarily mean that PCP signaling as we understand it in fly epithelia is at play, as these molecules may be functioning in other pathways (for review see, Goodrich and Strutt, 2011). It is well known that Fz and Dsh function in canonical Wnt signaling as well as in another non-canonical Wnt

pathway, the Wnt/Calcium signaling pathway (Sheldahl et al., 2003; Kohn and Moon, 2005). Furthermore some processes do not require the function of all core PCP components. Fmi, but not Fz or Dsh, is required for proper dendrite extension in the *Drosophila* (Gao et al., 2000); the vertebrate Fmi homolog Celsr regulates cell adhesion during zebrafish epiboly independent of other PCP components (Carreira-Barbosa et al., 2009) and Celsr3 and Fzd3a have been shown to function independently of Vangl2 in axon guidance (Chai et al., 2014; Qu et al., 2014; Chai et al., 2015). Additionally, an unexpected nuclear function has recently been attributed to vertebrate Pk (Mapp et al., 2011; Tao et al., 2012).

PCP Signaling in Vertebrates

Orthologs of all six core components of the PCP pathway, as well as each of the PCP effectors, are conserved in vertebrate genomes (See Table 1). Like fly epithelia, vertebrate epithelia also exhibit planar polarization, and the core PCP proteins have been demonstrated to mediate this polarity. The mechanosensory hair cells in the inner ear (Montcouquiol et al., 2003; Wang et al., 2005; Wang et al., 2006b), mammalian hair follicles (Guo et al., 2004; Devenport and Fuchs, 2008), the cells of the floorplate (Borovina et al., 2010) and the node/Kuppfer's vesicle (Antic et al., 2010; Hashimoto et al., 2010) all exhibit a form of planar polarization that requires the activity of PCP components (for review see, Wallingford, 2012). Core PCP proteins also function in diverse morphogenetic processes involving motile cells that display no obvious planar polarity. These include cell movements during gastrulation and neural tube closure; neuronal migration; axon outgrowth, branching and extension and cancer cell metastasis. The suite of phenotypes caused by loss of function of core PCP components mentioned here defines

"PCP processes" in vertebrates. Here I will focus on the role of PCP signaling molecules in directed cell motility.

Mutants in a number of other genes have a similar suite of phenotypes, implicating these genes in the PCP pathway: either in the establishment of polarized protein domains, in the reception or transduction of PCP signals or as downstream effectors that act upon the actin cytoskeleton to change cell shape or protrusive activity (See Table 2). With respect to PCP component trafficking, Sec24b, a component of the CopII coat protein complex, is required for trafficking of newly synthesized Vangl2, a trans-membrane protein, from the endoplasmic reticulum (Merte et al., 2010; Wansleeben et al., 2010; Giese et al., 2012). Recent work suggests that the adaptor protein Gipc1 and the unconventional MyosinVI interact with Vangl2 to promote its endocytic internalization and trafficking away from inappropriate membranes (Giese et al., 2012). Additionally, the GTP-binding protein, Arfrp1, and the clathrin adaptor complex-1 are required for Vangl2 transport from the trans Golgi network (Guo et al., 2013). PCP component localization is also maintained intracellularly by localized protein degradation: The E3 ubiquitin ligases Smurf1 and Smurf2 are recruited to sites of activated Dvl2 (a vertebrate Dishevelled homolog) where they target Pk1 for degradation (Narimatsu et al., 2009). As a result, Pk1 asymmetry and planar polarization is lost in the cochlea of Smurf1;2 mutant mice (Narimatsu et al., 2009).

A number of proteins have been implicated in the reception or transduction of Wnt signals that activate the PCP pathway in vertebrates and maintain asymmetric PCP protein localization. Glypican 4 (Knypek), a heparin sulfate proteoglycan, promotes PCP signaling through interactions with Wnt11 (Topczewski et al., 2001; Ohkawara et al., 2003). In mouse limb bud chondrocytes, the transmembrane receptor Ror2 binds Wnt5a and induces the

serine/threonine phosphorylation of Vangl2, increasing its proximal localization (Gao et al., 2011). Ptk7, a non-catalytic receptor tyrosine kinase, has been demonstrated to be required for planar polarization in a range of tissues and model vertebrates, however its mechanism is controversial (Lu et al., 2004; Shnitsar and Borchers, 2008; Yen et al., 2009; Glasco et al., 2012). In *Xenopus*, Ptk7 functions with the adaptor protein Rack1 in the recruitment of Dvl to activated Fz at the membrane (Shnitsar and Borchers, 2008; Wehner et al., 2011), however in mouse Ptk7 mutants Dvl membrane recruitment is normal but PCP is nevertheless disrupted (Yen et al., 2009; Lee et al., 2012). Recent experiments in the mouse cochlea have suggested that Ptk7 functions in parallel to the core PCP pathway in the regulation of MyosinII-dependent asymmetric tension in neighboring support cells, which aligns PCP in the sensory cells (Lee et al., 2012; Andreeva et al., 2014).

Several proteins have been identified as downstream components of PCP signaling molecules in vertebrates. The PDZ-domain containing protein Scribble, best known for its function in apico-basal polarization, also functions in vertebrate and fly PCP. Scrib interacts physically and genetically with Vangl, but how this interaction influences PCP is still not understood (Montcouquiol et al., 2003; Wada et al., 2005; Kallay et al., 2006; Courbard et al., 2009; Glasco et al., 2012). Core PCP components have been shown to regulate the apical accumulation and planar polarization of Rab11, which results in localized Myosin activation required for apical constriction in *Xenopus* gastrulation and neural plate folding (Ossipova et al., 2014; Ossipova et al., 2015). Daam1 is required for convergent extension during *Xenopus* gastrulation and directly binds Dsh and Rho in cultured cells (Habas et al., 2001). Rac1 is also hypothesized to act downstream of PCP signaling and in cultured cells Dsh and Vangl2 have been demonstrated to physically interact with Rac (Habas et al., 2003; Lindqvist et al., 2010).

Rho and Rac downstream of PCP signaling as well as other novel PCP effectors will be further discussed below.

Planar Cell Polarity and Cell Motility

The PCP Pathway Regulates the Collective Cell Movements of Convergent Extension

The best-characterized instances in which PCP signaling regulates the behaviors of motile cells is in the convergent extension (CE) movements of mesodermal cells during vertebrate gastrulation and of neuroepithelial cells during neural tube closure. CE is a process through which cells within a tissue intercalate to narrow and extend the tissue. Similar cell intercalations also drive the narrowing of the cochlea, kidney tube elongation and limb elongation (Wang et al., 2005; Gao et al., 2011; Lienkamp et al., 2012). Vertebrate homologs of Fz, Dsh, Vang, Pk, Celsr as well as Ptk7, Scrib and the PCP effector Fritz have all been implicated in CE movements (Sokol, 1996; Djiane et al., 2000; Wallingford et al., 2000; Wallingford and Harland, 2001; Darken et al., 2002; Goto and Keller, 2002; Jessen et al., 2002; Curtin et al., 2003; Takeuchi et al., 2003; Veeman et al., 2003b; Lu et al., 2004; Wang et al., 2006b; Kim et al., 2010). The non-canonical Wnts, Wnt5 and Wnt11 have also been shown to be essential (Moon et al., 1993; Heisenberg et al., 2000; Tada and Smith, 2000). Several excellent reviews have recently described convergent extension cell movements at the cellular and tissue level (for review see, Gray et al., 2011; Wallingford, 2012). Here I focus on recent findings that suggest how PCP molecules may bring about polarized cell movements.

Before the onset of CE, mesodermal cells in the *Xenopus* gastrula are un-polarized and extend and retract lamellipodia in all directions. At the onset of convergent extension

lamellipodia become polarized to medial and lateral membranes and make stable contacts specifically with mediolateral neighbors (Wilson and Keller, 1991; Shih and Keller, 1992). These stable lamellipodia allow cells to exert traction, resulting in cellular elongation and alignment to the mediolateral axis (Wilson and Keller, 1991; Shih and Keller, 1992). Live imaging studies of mesodermal cells in Xenopus and zebrafish have demonstrated that disruption of PCP components results in defects in the polarity and stability of lamellipodia, the mediolateral alignment and elongation of cells and in polarized cell intercalations (Wallingford et al., 2000; Goto and Keller, 2002; Jessen et al., 2002; Goto et al., 2005; Yin et al., 2008). It has also been shown that in the mouse neural tube neuroepithelial cells display polarized protrusive activity in the plane of the tissue and Ptk7 is required for this polarization (Williams et al., 2014). Convergence and extension of tissues not only occurs by way of medial migration of cells but also through the remodeling of cell-cell junctions as first described in *Drosophila* germband extension (Bertet et al., 2004; Blankenship et al., 2006). Mesodermal cells and neuroepithelial undergoing CE exhibit mediolateral and apical cell junctional remodeling events that drive cell neighbor exchanges which result in intercalation along the mediolateral axis (Nishimura et al., 2012; Shindo and Wallingford, 2014; Williams et al., 2014). The role of PCP in cell junctional remodeling will be discussed in detail below.

As noted above, PCP signaling has a significant effect on the behavior of cells undergoing convergent extension. Studies aimed at identifying factors that act as downstream effectors in this process, not surprisingly, uncovered factors that modulate the cytoskeleton, including the Rho family GTPases. Rho and Rac activity is required for *Xenopus* gastrulation (Tahinci and Symes, 2003) and were shown to be activated by Wnt11/Fz signaling in cell culture (Habas et al., 2003). Furthermore, a direct link between core PCP signaling and RhoA activation

has been established via the identification of the formin Daam1, which binds to both Dsh and RhoA to mediate Wnt11-induced activation of RhoA (Habas et al., 2001). The RhoA effector, Rho Kinase 2 (Rok2) also acts downstream of Wnt11 and has been shown to mediate cell elongation and mediolateral orientation of gastrulating mesoderm cells in zebrafish (Marlow et al., 2002). Other proteins implicated in PCP signaling during gastrulation include the Rho exchange factor (WGEF) (Tanegashima et al., 2008) and PKC delta (Kinoshita et al., 2003).

Recently the roles of some of the factors mentioned above and other recently identified factors that regulate CE downstream of PCP have been described in greater detail. Septins, which are cytoskeletal filament forming proteins thought to stabilize cell cortices, were shown to be required for cell junction remodeling during *Xenopus* mesodermal CE downstream of PCP signaling. Septin(Sept)2 was shown to functionally and physically interact with the PCP effector Fritz to promote CE and Sept2 and Sept7 were shown to control elongation of mesodermal cells along the mediolateral axis (Kim et al., 2010). In addition, Sept7 was shown to be required for the planar polarization of actomyosin-mediated cell cortex tension to mediolateral membranes (Shindo and Wallingford, 2014). It was shown that phosphorylated myosinII is enriched along mediolateral cell-cell junctions while Sept7 is enriched to mediolateral vertices where it maintains stable actin and consequently tension along mediolateral membranes. This localization of Sept7 was shown to require PCP signaling. These findings support a model in which PCP signaling leads to polarized tension and membrane shrinkage along mediolateral membranes, which in turn drives mediolateral intercalation and thus tissue elongation along the anterior-posterior axis.

It has also been demonstrated that neuroepithelial cells can utilize PCP signaling to direct convergent extension via intercalation-like cell rearrangements through coordinated contraction

of adherens junctions (AJs) along the mediolateral axis (Nishimura et al., 2012). Here it was shown that Celsr1 localizes at AJs in a planar-polarized pattern along the mediolateral axis of neuroepithelial cells where it recruits Dvl2, which locally activates Daam1. This in turn leads to the activation of PDZ-Rho-GEF and the recruitment of ROCKs, which results in localized activation of myosin. Actomyosin associated with mediolateral AJs contract producing cellular forces that results in the medial relocation of cells. In the mouse neural tube, Vangl2 was also shown to occupy a role in mediolateral biased cell neighbor exchanges (Williams et al., 2014). Interestingly, Rab11 was shown to be planar polarized specifically to the medial apical surface of neuroepithelial cells in the *Xenopus* neural tube where it is required for MyosinII activation and neural folding downstream of RhoA (Ossipova et al., 2014). Vangl2, Dsh and Diversin were shown to be required for this localization of Rab11 suggesting that directionally dependent membrane trafficking controlled be PCP signaling occupies a role in cell behavior during CE.

As I have discussed above, the asymmetric localization of PCP proteins to discrete sides of the cell is key in promoting morphological asymmetries in fly epithelia. Localizing PCP proteins in cells undergoing dynamic shape- and neighbor changes is more challenging, and has best been accomplished by imaging fluorescently tagged proteins that are mosaically expressed so that one cell's membrane can be distinguished from its neighbor's. This approach has shown that GFP-Pk is asymmetrically localized to the anterior membrane of zebrafish neural progenitors and mesodermal cells undergoing CE and that GFP-Dsh is localized, at least transiently, to the posterior membrane (Ciruna et al., 2006; Yin et al., 2008). In neuroectoderm and mesoderm undergoing CE movements, this polarized distribution of PCP proteins correlates with a slight posterior bias in the position of the microtubule organizing center (centrosome) (Sepich et al., 2011).

This glimpse of PCP component polarization suggests that it is possible that PCP protein localization influences cell polarity and behavior during convergent extension. Indeed, as discussed above PCP signaling was shown to control the mediolateral alignment of factors required for the proper orientation of cell intercalation events. However, it is not yet known how this localization is established. Polarization could be established via a Wnt signaling gradient, which seems likely since Wnt mutants have CE phenotypes. Additionally an unidentified chemokine gradient could be at play. It is also possible that this polarization is established via intercellular communication, which could help to set up coordinated cell polarity and thus directed movement. In support of this idea, chimeric analyses suggest that loss of core PCP protein activity in one cell can affect the mediolateral alignment of neighboring wild type cells (Jessen et al., 2002). In convergent extension, these interactions are likely between cells of the same type and it will be interesting to determine whether this type of communication can occur between different cell types.

Neural Crest Cell Migration

The neural crest (NC) is a multipotent cell population that is induced in the dorsolateral regions of the neural tube at the junction of neural and non-neural ectoderm. Once specified, NC cells undergo an epithelial to mesenchymal transition, delaminating from the dorsal neural tube and migrating throughout the embryo following well defined routes to their final destinations where they give rise to numerous neural, as well as non-neural tissues (for reviews see, Knecht and Bronner-Fraser, 2002; Mayor and Theveneau, 2013). Well described guidance molecules are involved in the patterning of NC cell migration. These are usually repulsive in nature and include the ligand receptor pairs Robo/Slit, Neuropilin/Semphorin and Ephrins/Eph (for review see,

Kuriyama and Mayor, 2008). One factor that has been proposed to attract NC cells is the chemokine Sdf1 (Belmadani et al., 2005; Olesnicky Killian et al., 2009). In addition to extracellular cues, cell-cell communication between NC cells is crucial for migration (Davis and Trinkaus, 1981; Teddy and Kulesa, 2004; Carmona-Fontaine et al., 2008). NC cells migrate collectively in cellular streams and cell-cell interactions between NC cells is thought to confer directionality during collective migration through contact inhibition of cell protrusions at cell-cell contacts, resulting in only leading cells extending stable lamellipodia in the direction of migration (Carmona-Fontaine et al., 2008; Theveneau et al., 2010). Below I will discuss a role for PCP in regulating NC migration and in establishing cell-cell contacts between NC cells.

Multiple PCP components have been shown to be required for NC cell migration in vertebrates. However, this role for PCP does not appear to be conserved in mammals (Hua et al., 2013; Pryor et al., 2014). Vangl2 is required for appropriate NC migration in the zebrafish trunk and disruption of PCP signaling severely inhibits cephalic NC migration in Xenopus and trunk NC crest migration in zebrafish (De Calisto et al., 2005; Matthews et al., 2008). Dvl function in NC migration has been proposed to require the vertebrate-specific PCP protein, Ptk7, which is required for migration and functions along with Fz7 to transport Dsh to the plasma membrane (Shnitsar and Borchers, 2008). Other non-core PCP components required for NC migration are Wnt11 and Wnt5a (De Calisto et al., 2005; Matthews et al., 2008; Banerjee et al., 2011). Interestingly, Wnt11 is expressed adjacent to migrating NC cells that express its putative receptor, Fz7. However, it is not clear whether Wnt11 plays an attractive role in NC migration as overexpression of Wnt11 in a ectopic location doesn't induce NC cells to migrate in an incorrect direction, but rather blocks their migration (De Calisto et al., 2005).

Another role for PCP signaling in the environment of migrating trunk NC cells has been established from studies of muscle-specific receptor kinase (MuSK), which like Fz is a transmembrane domain protein containing a conserved cysteine-rich domain that binds Wnt11r (Jing et al., 2009). In zebrafish *musk* is expressed in dorsal adaxial cells, which are required for segmental trunk NC migration (Honjo and Eisen, 2005), just adjacent to *wnt11r* expressing cells (Jing et al., 2009). In zebrafish *musk* mutants and *wnt11r* mutants, NC cells are no longer restricted to their segmental stream in the central somite and instead spread across the entire somite (Banerjee et al., 2011). Expressing dominant negative Dvl specifically in adaxial muscle cells results in the same migration defect (Banerjee et al., 2011), suggesting that the PCP signaling is not autonomous to the NC cells. These findings are intriguing because it suggests a requirement for PCP signaling in the environment. Perhaps this environmental signaling could act to polarize NC cell protrusions to coordinate migration into directed streams. A cell autonomous role for PCP signaling in regulating NC cell protrusive activity is discussed below.

A role for PCP signaling in contact inhibition of NC cell locomotion, which requires establishment of NC cell-cell contacts and is proposed to lead to local activation of RhoA, has been established (Carmona-Fontaine et al., 2008). Live imaging of wild type NC cells showed that only leading cells are highly polarized and extend protrusions in the direction of migration. However, consistent with earlier findings (De Calisto et al., 2005; Matthews et al., 2008), NC cells with disrupted PCP signaling were shown to crawl on top of one another, with both leading and trailing cells extending protrusions in all directions (Carmona-Fontaine et al., 2008). This was demonstrated in cultured *Xenopus* NC cells following expression of dominant negative Dvl or Wnt11 and *in vivo* in zebrafish trunk NC cells following morpholino knockdown of *vangl2*,

pk1 and *wnt11* and dominant negative Dvl expression (Carmona-Fontaine et al., 2008). Using fluorescence resonance energy transfer (FRET) biosensors for Cdc42, Rac and RhoA, it was shown that PCP signaling in NC cells likely regulates cellular protrusive activity by activating RhoA (Matthews et al., 2008). Activating PCP signaling in NC cells results in an increase in RhoA activation and inhibition of PCP signaling results in a decrease of RhoA activation both *in vitro* and in *Xenopus* embryos (Matthews et al., 2008). Additionally, RhoA activation was shown to increase in colliding cells with the site of cell-cell contact exhibiting highest activation levels (Carmona-Fontaine et al., 2008). Analysis of PCP protein localization *in vitro* and *in vivo* revealed that membrane localization of Dvl, Wnt11 and Fz7 is observed at sites of cell-cell contact, which is at the back of leading cells, and that Dvl becomes re-localized to the site of cell contact when two cells collide (Carmona-Fontaine et al., 2008). These findings suggest that cell-cell contact leads to the localized activation of PCP signaling, which polarizes the cell in the direction of migration by locally activating RhoA at the cell rear. Furthermore, inhibition of the downstream Rho effector Rock resulted in loss of contact inhibition (Carmona-Fontaine et al., 2008) and leads to increased Rac activation (Matthews et al., 2008). These findings demonstrate a requirement for RhoA activation in mediating contact inhibition and suggest that RhoA activation at the back of the cell may polarize protrusions by restricting Rac activation. Additionally, the actin binding protein Calponin2 (Cnn2) localizes to the leading edge of migrating NC cells in chicks and frogs and is required for NC migration downstream of Wnt11 and RhoA (Ulmer et al., 2013).

The study of PCP signaling in NC cells supports a role for PCP signaling between cells in promotion of directional migration as a homophilic interaction between NC cells was shown to be important in establishing cell polarization and migration direction and PCP signaling in the

cells surrounding migrating NC cells was shown to be necessary for migration. Additionally, the proteoglycan syndecan-4 is thought act in parallel to PCP signaling to mediate NC migration by inhibiting Rac activity at points of cell-cell contact (Matthews et al., 2008). The asymmetric localization of PCP components in leading cells suggests a role for cell-cell communication in establishing PCP localization, which is reminiscent of PCP localization in stable epithelia cells. Additionally, these studies further demonstrate a link between PCP signaling and modulation of the cytoskeleton in migrating cells. Importantly, it was demonstrated that cell-to-cell PCP signaling may function to localize RhoA activity and thus polarize cell protrusive activity in the direction of migration. However, even though PCP signaling surely occupies an important role in guiding NC migration, it does not exclude the possibility that extracellular factors such as Sdf1 are not involved in directing polarized NC migration.

PCP and the Migration of Non-Coherent Cells

Axon Guidance

A role for PCP pathway members in axon guidance was first uncovered when Fzd3 and Celsr3 were shown to be required for the formation of several major axon tracts in the mouse (Wang et al., 2002; Lyuksyutova et al., 2003; Tissir et al., 2005). Loss of either of these genes results in loss of the major axon tracts that connect the thalamus and the cortex and failure of commissure neurons in the spinal cord to extend anteriorly following midline crossing. Like Fzd3a and Celsr3, Vangl2 is required for the proper anterior-posterior (A-P) guidance of commissural axons in mice (Shafer et al., 2011). In addition, Fzd3a, Celsr3 and Vangl2 are required for the A-P guidance of monoaminergic neuron axons in the mouse brainstem, Vangl2

occupies a role in the guidance of mouse retinal ganglion cell axons, Fzd3 is required for guidance of several cranial nerves and outgrowth of motor neuron axons in the limb and Fzd3 and Celsr3 are required for the development of a number of forebrain tracts in the mouse (Fenstermaker et al., 2010; Hua et al., 2013; Hua et al., 2014; Leung et al., 2015). Recent loss of function studies suggested that Celsr2, Celsr3 and Fzd3 regulate axon guidance in the forebrain independently of Vangl2 and that previously described roles for Vangl2 in axon guidance may be due to a dominant effect of the *looptail* mutation (Qu et al., 2014). Celsr3 and Fzd3 were also suggested to act independently of Vangl2 during axon guidance of motor neurons in the hindlimb and in this instance Celsr3 and Fzd3 are thought to guide axons via an interaction with EphA-ephrinA reverse signaling (Chai et al., 2014). However, in support of a role for Vangl in axon guidance, loss of Vang function results in aberrant axon targeting and branching in the *Drosophila* mushroom body and loss of Vangl1 results in defective neurite outgrowth in *C. elegans* (Sanchez-Alvarez et al., 2011; Shimizu et al., 2011; Ng, 2012). Core PCP components Fz, Celsr and Dsh also function in axon targeting in the *Drosophila* mushroom body and Fz2 and Dsh were shown to be required for proper retinal axon targeting in the *Drosophila* visual system (Sato et al., 2006; Shimizu et al., 2011; Ng, 2012). Additionally, Pk1 and Dsh1 were shown to regulate neurite outgrowth in *C. elegans* (Sanchez-Alvarez et al., 2011). These studies suggest a conserved role for core PCP components in axon guidance

Wnt proteins have been indicated to act upstream of PCP signaling molecules to regulate axon guidance and outgrowth. Initial studies of axon growth in rat spinal cord explants showed that several Wnts, including Wnt4, Wnt5a, and Wnt7b can stimulate axon outgrowth (Lyuksyutova et al., 2003). Furthermore, blocking Wnt signaling disrupts A-P guidance of commissural neurons (Lyuksyutova et al., 2003). Recently, Wnt5a-stimulated axon outgrowth in

cultured commissural neurons was shown to be mediated by Fzd3 and to require Vangl2 (Shafer et al., 2011). Additionally, Wnt5a mutant mice show transient A-P guidance defects of monoamingergic neuron axons (Fenstermaker et al., 2010; Blakely et al., 2011). In cultured explants, Wnt5a attracts serotonergic axons and interestingly Wnt5a repels dopaminergic axons, while Wnt7b attracts dopaminergic axons (Fenstermaker et al., 2010). Fzd3 was further shown to mediate Wnt signaling, as dopaminergic axons from Fzd3 mutant mice explants do not respond to Wnt5a or Wnt7b (Fenstermaker et al., 2010; Blakely et al., 2011). In support of Wnt signaling guiding axon outgrowth *in vivo*, Wnt4a, Wnt5a and Wnt7b have been shown to be expressed in gradients along the anterior posterior axis (Lyuksyutova et al., 2003; Fenstermaker et al., 2010). Wnt5a and Wnt7a were shown to be required for A-P guidance of neurons in the chick spinal cord (Domanitskaya et al., 2010). In the chick, however, these Wnts are not expressed in a gradient and A-P axon guidance is thought to be directed by a gradient of the secreted frizzled related protein 1 (Sfrp1) which blocks the attractive effect of Wnt5a and Wnt7a (Domanitskaya et al., 2010). In the zebrafish, the Wnt11r receptor MuSK, introduced above, is required to organize the central muscle zone to spatially restrict axon growth cones, a process which requires functional Dvl in muscle fibers (Jing et al., 2009). Although Wnts are typically not thought to function in *Drosophila* PCP signaling, Wnt5 has also been shown to be required for axon targeting and branching in the *Drosophila* mushroom body and was also demonstrated to genetically interact with core PCP components in this process (Shimizu et al., 2011). Wnt5a has also been demonstrated to mediate cortical neuron axon repulsion via signaling through the atypical receptor tyrosine kinase (RYK) receptor, which evokes increased intracellular calcium (Keeble et al., 2006; Li et al., 2009; Li et al., 2010; Hutchins et al., 2012). In addition, atypical protein kinase C and phosphatidylinositol-3-kinases signaling was shown to be required for the

Wnt mediated attraction of commissural neurons independently of calcium (Wolf et al., 2008). Thus, Wnt mediates axon guidance through modulation of different downstream targets which may act in parallel to the PCP signaling pathway.

In cultured commissural neurons c-Jun N-terminal kinases (JNK), which was previously shown to act downstream PCP signaling in some contexts and as mentioned above to promote cell motility, is activated by Wnt5a and required for A-P guidance of commissural axons (Shafer et al., 2011). In rat spinal cord lysates increased JNK activation was observed in the ventral spinal cord where crossing occurs and the addition of JNK inhibitors to open-book explants randomized A-P targeting. Interestingly, JNK signaling in growth cones appears to be controlled by mutual antagonism between Vangl2 and Dvl1. Vangl2 was proposed to antagonize Dvl1 by interfering with Dvl1 mediated phosphorylation of Fzd3, which interferes with Fzd3 internalization. Furthermore, live imaging revealed that Vangl2 is found predominantly on the growth cone at stable or growing filopodia, where it is proposed to promote Fzd3 internalization and JNK signaling. Wnt5a was shown to promote Fzd3 endocytosis in commissural growth cones and this was shown to correlate with filopodia growth (Onishi et al., 2013). Here an unanticipated antagonism between Dvl1 and Dvl2 was identified in which Dvl2 blocks Dvl1 mediated phosphorylation of Fzd3 and thus membrane accumulation of Fzd3. In this study it was also shown that in a Wnt5a gradient more Fzd3 endocytosis occurs on the side of the growth cone facing higher Wnt5a, suggesting that spatial control of Fzd3 endocytosis may be part of the mechanism by which PCP signaling directs axon pathfinding.

As discussed above, PCP signaling molecules in axons may promote the establishment of asymmetric signaling, which in turn may activate downstream pathways such as JNK to promote directed growth. This further demonstrates that PCP signaling may activate different downstream

targets depending on the cell type, suggesting that PCP signaling molecules can have distinct functions that are context dependent. Interestingly, several Wnts are expressed in gradients along the path of growing axons and appear to be instructive in guiding outgrowth direction (Lyuksyutova et al., 2003; Fenstermaker et al., 2010). This is in contrast to the ubiquitous expression of *wnt5b* and *wnt11* observed in the domain of mesoderm and neuroectoderm cells undergoing convergence extension (Heisenberg et al., 2000; Kilian et al., 2003). Wnt5a influences the localization of PCP components in commissural axon growth cones (Shafer et al., 2011; Onishi et al., 2013) further supporting an instructive role for Wnts in establishing the direction of motility. The examination of PCP component localization in commissural neuron axons demonstrates that PCP components can regulate the localization and activity of each other similarly to what is observed in stationary epithelia. However, in contrast to studies in *Drosophila*, Vangl2 and Fzd3 colocalize and Vangl2 appears to promote Fzd3 signaling cell-autonomously. This suggests that the spatial relationship and therefore output of PCP signalling molecules varies between cell types. Furthermore, although localization is much more dynamic in the motile growth cone, Vangl2 was shown to be asymmetrically localized to stable and growing filopodia and Fzd3 at the tip of growing filopodia was shown to be internalized, suggesting that the localization of core PCP components may be an important factor in directing axons.

Cancer Cell Migration

Several core PCP proteins have been found to be overexpressed in a number of cancers and upregulation of these proteins is correlated with poor patient prognosis (Jessen, 2009). This is likely due to promotion of metastasis as a several studies have demonstrated a requirement for

PCP signaling in tumor cell motility. PCP signaling is required for breast cancer cell (BCC) migration and metastasis, as downregulation of Fzd6, Dvl1, Vangl2, Pk1 and Wnt11, severely reduces protrusive activity and migration of BCCs and Pk1 is required to promote BCC metastasis in a mouse model (Luga et al., 2012). In addition, Fzd2, Dvl1, Vangl1, Scrib, and Wnt5a have been shown to be required to promote cancer motility in a number of cancer cell lines (Yamamoto et al., 2010; Anastas et al., 2012; Gujral et al., 2014; MacMillan et al., 2014; Saxena et al., 2015). Interestingly, Vangl2 has also been shown to inhibit cancer cell motility as loss of Vangl2 in human fibrosarcoma cells actually promotes collective migration and tumor cell invasion (Cantrell and Jessen, 2010). In this instance, loss of Vangl2 results in increased levels of secreted and membrane bound matrix metalloproteinases.

PCP signaling components have been shown to interact with several novel pathways in cancer cell lines. In several cancer cell lines binding of Wnt5 to Fzd2 promotes motility, epithelial mesenchymal transition (EMT) and polarization of cells at the leading edge in scratch wound assays (Gujral et al., 2014). This activity of Fzd2, however, didn't appear to be regulated by activation of Dvl proteins. Instead, upon Wnt5 stimulation Fzd2 was shown to physically associate with and control the activity of Fyn, a Src family kinase, and Stat3, a transcription factor whose activity drives expression of EMT markers. PCP components could also act in parallel with or downstream of various proteins not generally considered to be part of the PCP signaling pathway. For instance, Sirtuin1 (Sirt1) deacetylase was shown to regulate Dvl1 levels and the association of Dvl1 with the Rac guanine nucleotide exchange factor, T-cell lymphoma invasion and metastasis 1 (Tiam1), which promotes activation of the Rac pathway and cancer cell motility (Saxena et al., 2015). Additionally, in breast cancer cells Vangl1, Scrib and Nitric oxide synthase 1 adaptor protein 1 (NOS1AP) were found in a complex using mass spectrometry

and were shown to partially colocalize to cell protrusions endogenously (Anastas et al., 2012). In this study these proteins were shown to be required to promote the migration of breast cancer cells and for proper polarization of migrating cells in scratch wound assays.

It has also been reported that signals from the cellular environment can activate PCP signaling and promote cancer cell migration (Luga and Wrana, 2013). It was shown that exosomes secreted by fibroblasts in the tumor microenvironment are internalized by BCCs, where they induce the mobilization of BCC produced Wnt11 to stimulate autocrine Wnt-PCP signaling (Luga et al., 2012). This is an interesting mechanism by which cells in the tumor environment promote migration of tumor cells by activating PCP signaling. In this study, analysis of the endogenous protein localization of Fzd6 and Dvl1 in BCCs revealed that upon stimulation by Wnt11 these proteins become enriched at the leading edge of cells protrusions. In contrast, endogenous Pk1 and ectopically expressed Flag-Vangl1 localize along the non-protrusive membrane at the base of protrusions, mutually exclusive of Fzd6. These results reveal an asymmetric distribution of Fzd6-Dvl1 and Vangl1-Pk1 with respect to cellular protrusions, in a manner that is analogous to the planar polarization of these protein complexes in epithelial cells. This is the first report of such a distribution in single motile cells. Interestingly, in cultured transformed lymphocytes fluorescently tagged Vangl2 was shown to localize to the trailing edge while Dvl3 was shown to localize to the leading edge of migrating cells (Kaucka et al., 2015). This demonstrates that PCP proteins display distinct localizations with regard to cellular protrusions and direction of migration in various cancer cell lines, further suggesting that PCP signaling can employ a range of mechanisms to promote cell migration.

A Role for PCP Signaling in Neuronal Migration

In the developing nervous system many neuronal populations migrate substantial distances from germinal zones to their final destinations where they carry out their specialized functions. Neurons can migrate radially, associated with glia, or tangentially, within the plane of the epithelium. The migration of facial branchiomotor (FBMNs) in the vertebrate hindbrain is useful model in which to study tangential migration. FBMNs are a subset of cranial branchiomotor neurons that originate ventrally in rhombomere (r)4 and undergo a highly stereotyped posterior migration to r6 and r7 (for review see, Chandrasekhar, 2004; Wanner et al., 2013). There they migrate dorso-laterally to form the facial motor nucleus whose axons exit the hindbrain in r4 and innervate muscles derived from the second branchial arch (Chandrasekhar, 2004). FBMNs migrate through the plane of the neuroepithelium adjacent to the floorplate in contact neuroepithelial progenitors as well as with other migrating neurons (Grant and Moens, 2010). Cells of both the neuroepithelium and the floorplate are highly polarized along their apico-basal and planar (anterior-posterior) (AP) axes (Ciruna et al., 2006; Borovina et al., 2010; Walsh et al., 2011).

Multiple PCP signaling components have been shown to be required for the tangential migration of FBMNs. The zygotic loss-of-function of the core PCP components Vangl2, Pk1b, Fz3a and Celsr2 all result in the complete failure of FBMN migration in the zebrafish (Bingham et al., 2002; Jessen et al., 2002; Carreira-Barbosa et al., 2003; Wada et al., 2006; Rohrschneider et al., 2007; Mapp et al., 2011). Additionally, Scribble (Scrib), which as mentioned above, in addition to its role in apico-basal polarity, is required for FBMN migration in the zebrafish (Wada et al., 2005). Similar roles for Vangl2, Fzd3, Pk1 and Celsr have been observed in murine FBMN migration (Vivancos et al., 2009; Qu et al., 2010; Yang et al., 2014). Nance-Horan

syndrome-like 1b (Nhsl1b), was also shown to be required for zebrafish FBMN migration (Walsh et al., 2011). Nhsl1b functions specifically in FBMN migration and not in other PCP dependent processes, physically and genetically interacts with Scrib in regulating FBMN migration and is localized to cell protrusions, suggesting that this protein functions as a neuron-specific PCP effector (Walsh et al., 2011). Similar to NHS homologs, zebrafish Nhsl1b contains a WAVE homology domain (WHD) found in WAVE (Wiskott-Aldrich syndrome protein family Verprolin-homologous) proteins (Brooks et al., 2010), which is essential for its function in FBMN migration, suggesting Nhsl1b is required for membrane dynamics (Walsh et al., 2011). Unlike the cell migrations mentioned above, screens have failed to identify a role for Wnts or other chemotactic cues in FBMN migration.

Studies aimed to identify the tissues in which PCP is required to promote FBMN migration has led to conflicting results. Initial chimeric analyses suggested that Vangl2, Fz3a, Celsr2 and Scrib primarily function non-cell-autonomously, in the migratory environment, to regulate FBMN migration (Bingham et al., 2002; Carreira-Barbosa et al., 2003; Qu et al., 2010; Walsh et al., 2011). In addition to their non-cell-autonomous roles in FBMN migration, a cell-autonomous role for Vangl2 and Scrib has been demonstrated (Walsh et al., 2011). However, Vangl2 was recently suggested to solely act in the floorplate to promote FBMN migration (Sittaramane et al., 2013). Pk1b and Nhsl1b are not expressed in the migratory environment and are exclusively required cell-autonomously for FBMN migration (Mapp et al., 2010; Walsh et al., 2011).

Although it is clear that many components of the PCP pathway are required for FBMN migration, how these components regulate this highly dynamic process is unknown. How PCP controls directed cell movements is best, though incompletely, understood in coherently

migrating cells such as those undergoing convergent extension. How independently migrating cells utilize PCP signaling to direct migration is less well understood and many studies aimed at addressing this question have been conducted *in vitro*. The goal of my work was to better understand how PCP can regulate the migration of non-coherent cells and to determine how PCP signaling between different cell types, the migrating neurons and the cells through which they migrate, can modulate migratory cell behaviors. Using the migration of FBMNs in the zebrafish hindbrain as a model enabled us to study this process live and *in vivo*.

We first defined the cell types participating in PCP signaling during FBMN migration since previous studies have yielding conflicting results. Using the Gal4/UAS system to systematically disrupt PCP in a cell-type and rhombomere-specific manner, we demonstrate the dual requirement for PCP within FBMNs and the planar-polarized r4 neuroepithelial environment in which they arise, and identify reciprocal PCP-dependent interactions between FBMNs and the planar-polarized floorplate as being sufficient, though not required, to promote migration. Since cell migration results from the contact-dependent stabilization of cellular protrusions and PCP signaling is known to regulate actin dynamics, I examined the protrusive activity of single FBMNs using high-resolution single-cell time-lapse microscopy in chimeric embryos and demonstrate opposing functions for the PCP core components Fzd3a and Vangl2 in regulating FBMN filopodial protrusive activity *in vivo*. Within FBMNs I show that Fzd3a is required to stabilize filopodia while Vangl2 has an antagonistic, destabilizing role. However, in the migratory environment I show that Fzd3a is required to destabilize filopodia while Vangl2 has a stabilizing role. In spite of having antagonistic roles at the cellular level, Vangl2 and Fzd3a mutants have the same FBMN migration phenotype. These findings are thus reminiscent of the intracellular antagonistic versus intercellular stabilizing roles that core PCP proteins perform in

stably polarized epithelia. Consistent with a role for Vangl2 in regulating filopodial dynamics, I show that Vangl2 localizes transiently to the tips of retracting FBMN filopodia; consistent with a role for Vangl2 and Fzd3a in the microenvironment, I show planar polarized localization of these proteins in the adjacent floorplate. Together, these findings support a model in which canonical interactions between PCP components within FBMNs and between the FBMNs and their planar polarized neuroepithelial environment promotes migration via the selective stabilization of FBMN filopodia.

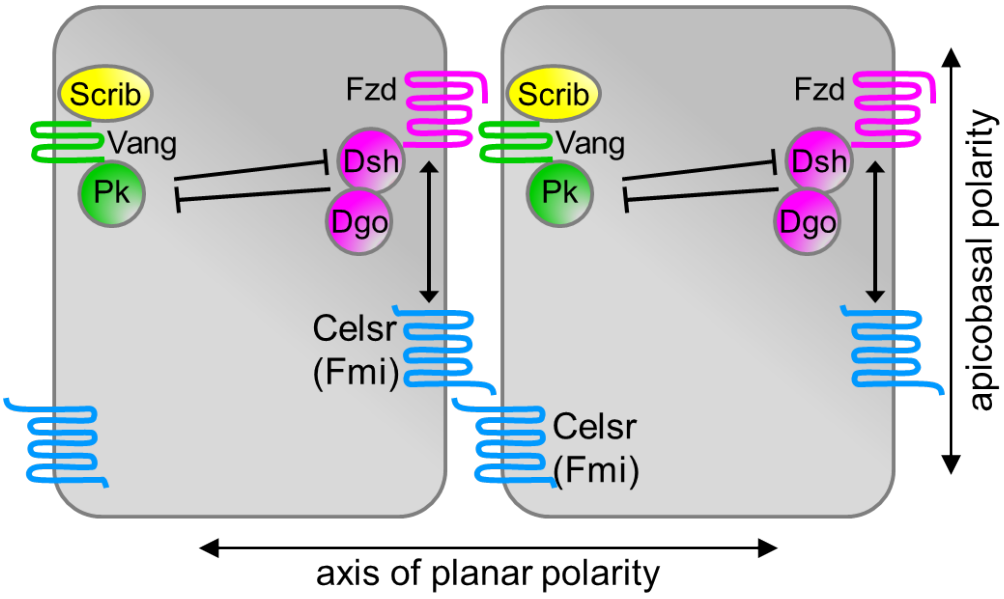

Figure 1: Localization of Core Planar Cell Polarity (PCP) components.

Lateral view of two cells that have established asymmetric localization of the core PCP components. Outlined are the molecular interactions between these components that are used to establish molecular asymmetry.

Table 1: PCP signaling components in *Drosophila* and Vertebrates

	Drosophila	Vertebrates	Protein Type	Vertebrate Processes
Core PCP components	Van Gogh/ Strabismus (Vang/Stbm)	Van Gogh Like 1 (Vangl1) Van Gogh Like 2 (Vangl2)	Four-pass transmembrane (TM), with intracellular PDZ binding domain	Facial Branchiomotor (FBMN) migration (Bingham et al., 2002; Jessen et al., 2002; Vivancos et al., 2009) Gastrulation (Darken et al., 2002; Goto and Keller, 2002; Jessen et al., 2002; Park and Moon, 2002) Neural tube closure (Kibar et al., 2001; Murdoch et al., 2001; Kibar et al., 2007) Inner ear development (Montcouquiol et al., 2003; Wang et al., 2005) Epidermal hair polarity (Devenport and Fuchs, 2008) Neural Crest (NC) migration (Carmona-Fontaine et al., 2008; Matthews et al., 2008) Growth cone guidance (Fenstermaker et al., 2010; Shafer et al., 2011) Limb bud elongation (Gao et al., 2011) Orientation of motile cilia (Mitchell et al., 2009; Borovina et al., 2010; Song et al., 2010; Vladar et al., 2012; Boutin et al., 2014) Orientated cell division (Gong et al., 2004)
	Frizzled (Fzd, Fz)	Frizzled 2 (Fzd2) Frizzled 3 (Fzd3) Frizzled 6 (Fzd6) Frizzled 7 (Fzd7)	Seven-pass transmembrane receptor	FBMN migration (Wada et al., 2006; Vivancos et al., 2009) Gastrulation (Deardorff et al., 1998; Djiane et al., 2000; Wallingford et al., 2001) Inner ear development (Montcouquiol et al., 2006; Wang et al., 2006b) Neural tube closure (Wang et al., 2006b) Orientation of motile cilia (Mitchell et al., 2009; Boutin et al., 2014) NC migration (Carmona-Fontaine et al., 2008) Epidermal hair polarity (Guo et al., 2004) Growth cone guidance (Fenstermaker et al., 2010; Shafer et al., 2011)
	Flamingo/ Starry Night (Fmi/Stan)	Celsr1 Celsr2 Celsr3	Seven-pass TM with extracellular cadherin repeats	FBMN migration (Qu et al., 2010) Inner ear development (Curtin et al., 2003) Neural tube closure (Curtin et al., 2003) Epidermal hair polarity (Devenport and Fuchs, 2008) Ciliogenesis (Tissir et al., 2010; Boutin et al., 2014) Growth cone guidance (Fenstermaker et al., 2010; Shafer et al., 2011)
	Dishevelled (Dsh)	Dishevelled 1 (Dvl1) Dishevelled 2 (Dvl1) Dishevelled 3 (Dvl1) (Dsh zebrafish, Xenopus)	Cytoplasmic, contains DEP, DIX and PDZ domains	Gastrulation (Sokol, 1996; Tada and Smith, 2000; Wallingford et al., 2000; Wallingford and Harland, 2001) Inner ear development (Hamblet et al., 2002; Wallingford and Harland, 2002; Wang et al., 2006a) Neural tube closure (Wang et al., 2005; Etheridge et al., 2008) Orientation of motile cilia (Mitchell et al., 2009; Hashimoto et al., 2010) NC migration (De Calisto et al., 2005) Ciliogenesis (Park et al., 2008) Orientated cell division (Gong et al., 2004)
	Prickle (Pk)	Prickle 1 (Pk1) Prickle 2 (Pk2)	Cytoplasmic, LIM domain	FBMN migration (Carreira-Barbosa et al., 2003; Rohrschneider et al., 2007) Gastrulation (Takeuchi et al., 2003; Veeman et al., 2003b) NC migration (Carmona-Fontaine et al., 2008)
	Diego (Dgo)	*Inversin (Inv) *Diversin (Div)	Cytoplasmic, ankyrin repeats	Left-right patterning, Kidney development, Gastrulation (Simons et al., 2005) Gastrulation, heart formation (Moeller et al., 2006) * functions in canonical Wnt signaling (Schwarz-Romond et al., 2002; Simons et al., 2005)
Effectors	Fuzzy (Fy)	Fuzzy (Fy/Fuz)	Predicted TM domain	Ciliogenesis, neural tube closure (Park et al., 2006; Gray et al., 2009; Heydeck et al., 2009; Heydeck and Liu, 2011)
	Inturned (In)	Inturned (In)	Predicted TM domain	Ciliogenesis, neural tube closure (Park et al., 2006; Heydeck and Liu, 2011)
	Fritz (Frtz)	Fritz (Ftz)	WD-40 domains	Ciliogenesis , Gastrulation(Kim et al., 2010)
Parallel signaling molecules	Fat (Ft)		TM cadherin	Inner ear development, neural tube closure, kidney development, FBMN migration (Saburi et al., 2008; Zakaria et al., 2014)
	Dachsous (Ds)		TM cadherin	Inner ear and kidney development, FBMN migration (Mao et al., 2011; Zakaria et al., 2014)
	Four-jointed(Fj)	Four-jointed (Fjx)	Glogi kinase	Kidney development (Saburi et al., 2008)

Table 2: Vertebrate Specific PCP Signaling Molecules

	Protein Type	Developmental Processes
Wnt5	Secreted lipid-modified glycoprotein	Gastrulation (Moon et al., 1993; Wallingford et al., 2001) Neural tube close, inner ear development (Kilian et al., 2003; Qian et al., 2007) Neural Crest (NC) migration (Matthews et al., 2008) Axon guidance (Blakely et al., 2011)
Wnt7	Secreted lipid-modified glycoprotein	Hair cell polarization (Dabdoub et al., 2003) Axon guidance (Fenstermaker et al., 2010)
Wnt11	Secreted lipid-modified glycoprotein	Gastrulation (Heisenberg et al., 2000; Tada and Smith, 2000) NC migration (De Calisto et al., 2005)
Knypek/Glypican 4 (Kny/Gpc4)	Heparan sulphate proteoglycan	Gastrulation (Topczewski et al., 2001; Ohkawara et al., 2003)
Protein tyrosine kinase 7 (PTK7)	Transmembrane tyrosine kinase receptor	Inner ear development, Neural tube closure (Lu et al., 2004; Lee et al., 2012) NC migration (Shnitsar and Borchers, 2008)
Scribble (Scrib)	Scaffold Protein with Leucine-rice repeat (LRR) domain and 3 PDZ domains	FBMN migration (Wada et al., 2005; Vivancos et al., 2009) Inner ear development (Montcouquiol et al., 2003)
Sec24b	Part of COPII coat protein complex	Inner ear development, Neural tube closure (Merte et al., 2010; Wansleeben et al., 2010)
Ror	Single pass transmembrane with tyrosine kinase domain	Limb bud elongation (binds Wnt5a) (Gao et al., 2011)
Dishvelled associated activator of morphogenesis (Daam)	Formin	Gastrulation (Habas et al., 2001)
Gipc1	PDZ domain containing adaptor protein	Hair cell polarization (Giese et al., 2012)
Smurf1/Smurf2	E3 ubiquitin ligase	Inner ear development, Neural tube closure (Narimatsu et al., 2009)

Chapter 2: Materials and Methods

Ethics Statement

All experiments involving zebrafish (*Danio rerio*) followed the regulatory standards and guidelines of the Fred Hutchinson Cancer Research Institute Institutional Animal Care and Use Committee (IACUC#1392).

Zebrafish Lines and Maintenance

All animals were maintained according to standard procedures (Westerfield, 1993) and staged as previously described (Kimmel et al., 1995). All mutant lines used were previously described and are registered at The Zebrafish International Resource Center (ZIRC): *fzd3a*^{rw689} (*olt*^{rw689}) (Wada et al., 2006), *prickle1b*^{fh122} (Mapp et al., 2011), and *vangl2*^{m209} (*tri*^{m209}) (Jessen et al., 2002). Previously described transgenic lines used were as follows: *Tg(isl1:GFP)rw0* (Higashijima et al., 2000), *Tg(isl1CREST-hsp70l:mRFP)fh1* (Grant and Moens, 2010), *TgBAC(hoxb1a:RFP)fh3* (Grant and Moens, 2010), *Tg(egr2b:KalTA4)* (Distel et al., 2009) and *Tg(hoxb1a(β-globin):Gal4VP16)um60* (Choe et al., 2012).

Cloning and transgenic line generation

The following transgenic lines were generated for this study: *Tg(shh:Gal4VP16)fh445*, *Tg(isl1:Gal4VP16)fh452*, *Tg(isl1-hsp70:mTFP)fh350*, *Tg(isl-hsp70:dvl-DEP-GFP)fh444*, *Tg(10XUAS:xdd1-GFP)fh446*, *Tg(10XUAS:fzdΔC-GFP)fh447* and *Tg(10XUAS:GFP-vangl2)fh453*. The Gal4VP16 sequence was obtained from the Nonet Lab (http://pcg.wustl.edu/nonetlab/ResourcesF/Zebrafish.html) and the 10XUAS plasmid was obtained from the Tol2 kit

(http://tol2kit.genetics.utah.edu/index.php/List_of_entry_and_destination_vectors) (Kwan et al., 2007). The mTFP construct was obtained from Alleleustrious, Inc (Cat# ABP-FP-TFA1000). To generate *Tg(shh:Gal4VP16)fh445*, the ar-B enhancer element of zebrafish *sonic hedgehog (shh)* (Muller et al., 1999; Ertzer et al., 2007) was amplified from a plasmid (gift from Uwe Strähle). For the Gal4 lines, the *shh* and *isl1* enhancers were inserted upstream of the *gata2* minimal promoter element (Meng et al., 1997). Vectors containing *xdd1* and full-length *Xenopus dvl* were obtained from the Moon Lab. Transgenic elements were cloned using the Gateway® (Life Technologies) system using the primer sequences listed in Table 1. Final DNA constructs were assembled in the pDESTpBHR4R3 plasmid (gift from the Brockerhoff Lab) or the CG5 Tol2 expression vector (Kwan et al., 2007). Transgenic embryos were generated by Tol2 transposase RNA co-injection with each plasmid at the single cell stage (Kawakami et al., 2000).

Cell Transplantation

Chimeric embryos were generated by transplantation at the blastula or gastrula stage as previously described (Carmany-Rampey and Moens, 2006; Walsh et al., 2011). To track transplanted cells, donor embryos carrying the *Tg(isl1:GFP)rw0*, *Tg(isl1:mRFP)fh1* or *Tg(isl1:mTFP)fh350* transgene were injected with 1% cascade blue-dextran or rhodamine-dextran (for live imaging) and 1% biotin-dextran (for imaging after fixation) (10,000 mw, Life Technologies). Host embryos were then processed and imaged for all donor-derived cells, donor-derived FBMNs or floorplate cells, and host FBMNs. Host and donor embryo genotypes were identified either by observing body axis elongation defects (for *vangl2* mutant hosts), by examining FBMN location at 48hpf or by genotyping (for *fzd3a* mutant hosts).

Whole-mount immunohistochemistry

Anesthetized embryos were fixed in 2% trichloroacetic (TCA) acid for 3 hours or 4% paraformaldehyde (PFA)/ 4% sucrose in PBS for 1 hour at room temperature. Fixed tissue was washed in PBS + 0.5% TritonX100 followed by standard blocking and antibody incubations. Following staining, brain tissue was dissected, cleared step-wise in a 25%, 50%, 75% glycerol series and mounted for confocal imaging. The following antibodies were used: rabbit anti-zebrafish Vangl2 (1:250, Anaspec Cat# AS-55659), mouse anti-islet1 39.4D5 (1:10, Developmental Studies Hybridoma Bank); chicken anti-GFP (1:500, Abcam Cat# ab13970); rabbit anti-ZO-1 (1:1000, Zymed Cat# 61-7300); mouse anti-Cc2d2a (1:100, (Bachmann-Gagescu et al., 2011)); rabbit anti-RFP (1:1000, Abcam Cat# ab62341). For analysis of chimeric embryos after fixation, host embryos were additionally stained with a fluorescently conjugated streptavidin (Life Technologies Cat# S32351) to enhance the detection of Biotin-Dextran-containing donor-derived cells.

Primary Cell Culture

Primary cultures of FBMNs were prepared from 24 hour post fertilization *Tg(isl1:mTFP)*; *Tg(hoxb1a:RFP)* embryos. The hindbrains of embryos were microdissected and dissociated as previously reported (Andersen, 2001). Cells were plated on a chambered coverglass (Sigma Z734756) coated with 5µg/mL poly-D-lysine (Sigma L8021) and 5µg/mL laminin (Sigma L2020) at a density of 4-5 hindbrains per 1.7 cm^2. FBMNs were distinguished from other *Tg(isl1:mTFP)*-expressing hindbrain motor neurons by virtue of *Tg(hoxb1a:RFP)* expression, which is restricted to hindbrain r4 and r4-derived neurons. Live imaging of explanted neurons was performed 5 hours after plating.

Imaging and data analysis

Imaging was performed using a Zeiss 700 confocal microscope or a Zeiss spinning disc microscope with a QuantEM EMCCD camera for live time-lapse imaging. For timelapse imaging, Z-stack images at 1µm steps were captured every 30 seconds for 15 minutes for *in vivo* time-lapse images and every 5 seconds for 10 minutes for cultured neurons. Filopodia were defined as long thin protrusions, less than 0.2 µm in diameter and more than 0.75 µm in length, measured from the cell body margin to the protrusion tip. *In vivo* filopodia lengths, lifetimes and fluorescent intensities of mRFP and GFP-Vangl2 were quantified using Zeiss Zen 2012 software. For cultured neurons, filopodium quantification was performed semi-automatically using Imaris FilamentTracer software (http://www.bitplane.com/imaris/filamenttracer). Mean anti-Vangl2 fluorescent intensity for all cell membranes were measured in user-drawn regions of interest using Zeiss Zen 2011 software. Graphs were generated and statistics were computed using GraphPad Prism software. Figure images were created using Adobe Photoshop and Adobe Illustrator.

Chapter 3: PCP Signaling is required within FBMNs and in their Rhombomere 4 Neuroepithelial Environment for Migration

FBMN-restricted expression of PCP-specific dominant negatives disrupts FBMN migration

Initial chimeric analyses suggested that the PCP components Vangl2, Fzd3a, Celsr2 and Scrib primarily act non-cell-autonomously to regulate FBMN migration (Jessen et al., 2002; Wada et al., 2005; Wada et al., 2006). A cell-autonomous role for Vangl2 and Scrib in FBMN migration has been demonstrated (Walsh et al., 2011), but refuted by others (Sittaramane et al., 2013). To determine whether PCP signaling is required cell-autonomously within FBMNs for their migration, we expressed a dominant negative (DN) form of the PCP core component Dvl specifically in branchiomotor neurons using the *islet-1* (*isl1*) CREST enhancer (Fig. 2A-C) (Higashijima et al., 2000). Dvl is the branching point between multiple Wnt signaling pathways, and the overexpression of its individual domains exert pathway-specific DN properties (Boutros et al., 1998). Work in multiple vertebrate systems has demonstrated that Xdd1 and Dvl-DEP, two truncated forms of Dvl, act as PCP-specific DNs (Sokol, 1996; Tada and Smith, 2000; Wallingford et al., 2000).

We raised stable *Tg(isl1:Dvl-DEP-GFP)* zebrafish in which FBMNs express Dvl-DEP-GFP. In wild type embryos, FBMNs fully migrate to r6 by 48 hours post fertilization (Fig. 2B). However Dvl-DEP-GFP expressing FBMNs largely fail to migrate, with 31/35 of *Tg(isl1:Dvl-DEP-GFP)* embryos displaying FBMN migration defects where most FBMNs (>75%) remain in in r4 (Fig. 2C). This demonstrates that PCP signaling within FBMNs is required for their migration.

Chimeric Analysis Demonstrates a Cell-autonomous Role for Fzd3a in FBMN migration

To further confirm this, and to test specifically whether the core transmembrane PCP component Fzd3a, like Vangl2 (Walsh et al., 2011), is required within FBMNs for migration, we used chimeric analysis to assess the ability of *fzd3a*^{rw689} mutant FBMNs to migrate in a normal planar polarized neuroepithelium. In these experiments we prevented host FBMN migration using a *pk1b* morpholino since it is well known that migrating FBMNs can carry other FBMNs with them independent of PCP signaling, complicating the interpretation of chimeras (Walsh et al., 2011; Wanner and Prince, 2013). *pk1b* morphants precisely phenocopy *pk1b* mutants in which FBMNs fail to migrate even though the surrounding neuroepithelium can support wild type FBMN migration (Rohrschneider et al., 2007; Mapp et al., 2011; Walsh et al., 2011). While 70.9% of wild type FBMNs migrate out of r4 in a *pk1b* morphant environment, only 19% of *fzd3a* mutant FBMNs do so (Fig. 3). This suggests that Fzd3a is required within FBMNs for migration. Together, the disruption of migration due to FBMN-restricted DN expression, chimeric analysis of *fzd3a* mutant FBMNs and previous chimeric analysis of *vangl2*^{m209} mutant FBMNs (Walsh et al., 2011) confirms a FBMN-autonomous requirement for PCP signaling in migration.

The non-autonomous requirement for PCP signaling in FBMN migration localizes to r4

While these data support a cell-autonomous requirement for PCP signaling in FBMN migration, PCP signaling in FBMNs is not *sufficient* for their migration. Indeed, a non-autonomous requirement for PCP signaling in FBMN migration has been well established in chimeras in which wild type FBMNs are unable to migrate in *vangl2*, *fzd3a*, *celsr2* or *scrib* mutant hosts (Jessen et al., 2002; Wada et al., 2005; Wada et al., 2006; Walsh et al., 2011). Since

PCP is a cell-contact mediated signaling pathway in which the same transmembrane protein components are required in both contacting cells (Goodrich and Strutt, 2011), an attractive hypothesis is that FBMNs receive PCP cues from cells in their environment that promote or direct their migration. Thus we sought to determine where PCP signaling is required in the FBMN migratory path for migration.

To block PCP signaling in distinct compartments of the hindbrain, we used the Gal4/UAS system to drive rhombomere-restricted expression of Xdd1-GFP as well as a C-terminally truncated Fzd3a, Fzd3aΔC-GFP, that lacks its cytoplasmic region, which has been shown to function as a potent PCP DN tool in zebrafish (Wada et al., 2006). We used *Tg(egr2b:KalTA4)* to drive expression throughout r3 and r5 starting at 12 hpf (Distel et al., 2009) and *Tg(hoxb1a:Gal4)* (Choe et al., 2012) to drive expression throughout r4 starting at 10 hpf (Fig. 2A). Expression of Xdd1-GFP or Fzd3aΔC-GFP along the migration path in the r5 neuroepithelium does not affect migration (Fig. 2D,E). In contrast, r4-restricted expression of Xdd1-GFP or FzdΔC-GFP completely blocks FBMN migration (Fig. 2F,G). This suggests that PCP signaling is required at the onset of, but not throughout FBMN migration.

It was not surprising that that FBMNs fail to migrate in *Tg(hoxb1a:Gal4)*; *Tg(UAS:DN-GFP)* embryos given that FBMNs arise in r4, and thus express *hoxb1a* throughout their early development, and we had already shown a cell-autonomous requirement. To assess whether PCP signaling plays a role in the r4 neuroepithelium outside of FBMNs, we transplanted wild type *Tg(isl1:mRFP)* donor FBMNs into the presumptive ventral hindbrain of *Tg(hoxb1a:Gal4)*; *Tg(UAS:Xdd1-GFP)* embryos and assessed the positions of donor-derived FBMNs at 48 hpf. In control hosts, 87% (328/378) of wild type donor-derived FBMNs migrated out of r4 (328/378). In contrast, in hosts expressing Xdd1-GFP in r4, only 17% (33/190) of donor-derived wild type

FBMNs migrate out of r4 (Fig. 2H-J). Thus, expression of Xdd1-GFP throughout r4 significantly hinders wild type FBMNs from initiating migration ($p<0.0001$, $\chi^2=207.8$) (Fig. 2J). This demonstrates that the non-autonomous requirement for PCP signaling for FBMN migration resides in r4.

Floorplate PCP can support, but is not required for FBMN migration

FBMNs migrate in the ventral neural tube adjacent to the floorplate (Fig. 2A, (Wada et al., 2006; Grant and Moens, 2010)) making the floorplate a potential source of PCP signaling for FBMN migration. A recent report found that floorplate expression of Vangl2 is both necessary and sufficient for FBMN migration (Sittaramane et al., 2013). Here, to investigate whether PCP signaling in the floorplate is required for FBMN migration, we generated a *Tg(shh:Gal4)* line (see methods) to drive Xdd1-GFP or Fzd3aΔC-GFP expression in the notochord and floorplate (Fig. 4A-C). In order to determine if dominant negative expression does indeed disrupt floorplate planar polarity, we quantified the anterior-posterior position of the basal body in single floorplate cells as the ratio of its distance from the anterior membrane to the full anterior-posterior cell length (Fig. 4G). Basal bodies in wild type floorplate cells are planar polarized to the posterior membrane (average position= 78% of cell length, (Walsh et al., 2011)). Conversely, basal body planar polarization is significantly disrupted in floorplate cells expressing Xdd1-GFP or Fzd3aΔC-GFP (average position = 63% and 59% of cell length respectively; Fig. 4D-F, H). By comparison, floorplate cells in *vangl2* mutants display a complete loss of basal body planar polarity (average position = 47% of cell length on average, (Walsh et al., 2011)) (Fig. 4). Although DN expression in the floorplate disrupted its planar polarity, FBMN migration occurred normally (Fig. 5A,B). This suggests that PCP signaling in the floorplate is not required

for FBMN migration. Loss of PCP in the floorplate might be compensated for by other planar polarized cells in the r4 neuroepithelial environment (Ciruna et al., 2006).

Floorplate PCP could nevertheless be sufficient for FBMN migration as has been suggested (Sittaramane et al., 2013). We tested the sufficiency of Vangl2 in the floorplate for FBMN migration in two ways. First, we expressed a GFP-Vangl2 fusion protein specifically in the floorplate of *vangl2* mutants and wild type siblings using stable *Tg(shh:Gal4)* driver and *Tg(UAS:GFP-Vangl2)* transgenic lines (*vangl2*$^{m209/m209}$*; Tg(shh:Gal4); Tg(UAS:GFP-Vangl2)*). Although GFP-Vangl2 was expressed broadly in the floorplate in these otherwise mutant embryos starting at 14 hpf, and exhibits planar-polarized localization (Fig. 5 and see below), it neither disrupted FBMN migration in a wild type embryo nor rescued migration in a *vangl2* mutant embryo (Fig. 5C,D). Secondly, we introduced wild type cells into the floorplate of *vangl2* mutants by transplantation at the blastula stage. We never observed rescue of host FBMN migration *vangl2* mutant *Tg(isl1:mRFP)* hosts with wild type donor-derived cells in the hindbrain floorplate (Fig. 5E). This includes 9 cases with 10 or more wild type floorplate cells in rhombomere 4. This is contrary to the findings of Sittramane et al. (2013) who found that a single wild type floorplate cell in r4 of a *vangl2* mutant could rescue FBMN migration. Together, our findings strongly suggest that Vangl2 function in the floorplate is not sufficient for FBMN migration. The rescue of FBMN migration observed by Sittaramane et al. (2013) may have been due to broader expression of Vangl2. While we observed nice restricted floorplate and notochord expression using the shh:Gal4 driver (Fig. 4A-C), they used the Tol2 gene trap line SAGFF187A, which drives expression in tissues besides the floorplate (Asakawa et al., 2008; Koide et al., 2009). It is also possible that the isl1:GFP positive neurons Sittaramane et al. (2013) observed in r6 of vangl2 mutants are not FBMNs but glossopharyngeal (nIX) neurons. These

neurons are born in r6 and migrate to r7 (Chandrasekhar et al., 1997). In vangl2 mutants the cell bodies of nIX neurons are mislocalized to r6, suggesting that Vangl2 is also required for nIX neuron migration (Bingham et al., 2002). Thus, these neurons could be mistaken for migrated FBMNs in a vangl2 mutant.

In these transplant experiments we noted that FBMNs as well as floorplate cells differentiate from donor-derived cells. This is not unexpected, given the close proximity of floorplate and branchiomotor neuron progenitors in the early embryo (Xiong et al., 2013). Interestingly, we observed that unlike the mutant host FBMNs, wild type donor-derived FBMNs sometimes migrate (Fig. 5F), and their ability to do so correlates with the number of wild type cells in the hindbrain floor plate (R^2=0.244; p=0.005). We conclude that Vangl2 function in the floorplate is not sufficient for FBMN migration, but that Vangl2 function in the floor plate can support the migration of wild type FBMNs. Taken together, we conclude that the floorplate can serve as a source of PCP signals for FBMN migration, but other cells in the r4 environment, which are also planar polarized (Ciruna et al., 2006) can compensate for the loss of normal floorplate PCP signaling.

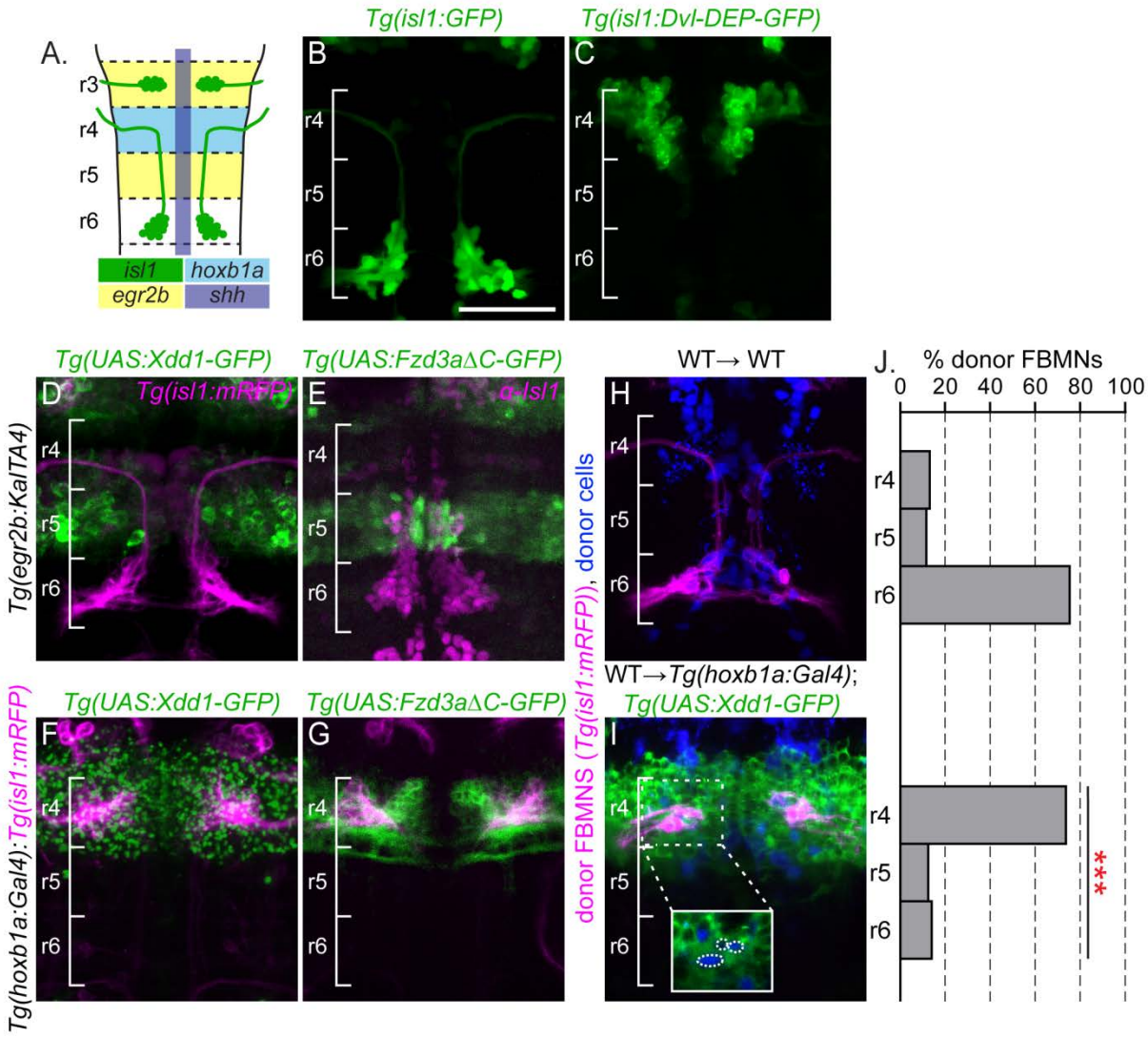

Figure 2: PCP signaling is required within FBMNs and in their r4 environment.

(A) Schematic showing a dorsal view of a 48 hours post fertilization (hpf) zebrafish hindbrain with anterior to the top. Facial Branchiomotor neurons (FBMNs) (green) migrate posteriorly from rhombomere (r) 4 to r6, leaving a trailing axon that exits from r4. The enhancer element *islet-1* (*isl1*) CREST drives expression in branchiomotor neurons (green); the *hoxb1a* element drives expression in r4 (light blue); *egr2b* drives expression throughout r3 and r5 (yellow) and *shh* drives expression in the floorplate (purple). (B-D,F-I) Live or (E) fixed confocal images showing dorsal views of the hindbrain of 48 hpf embryos with anterior to the top. Brackets mark

rhombomere (r) position. (B) *Tg(isl1:GFP)* expression in a wild type embryo at 48 hpf. (C) *Tg(isl1:Dvl-DEP-GFP)* embryo with unmigrated Dvl-DEP-GFP-expressing FBMNs in r4. (D-I) FBMNs (magenta) are either expressing *Tg(isl1:mRFP)(D,F-I)* or are stained with anti-Isl1 (E). (D,E) *Tg(egr2b:KalTA4)*-driven expression of *Tg(UAS:Xdd1-GFP)* (D) and *Tg(UAS:Fzd3a*Δ*C-GFP)* (E), throughout r3 and r5 does not block FBMN migration. (F,G) *Tg(hoxb1a:Gal4)*-driven expression of *Tg(UAS:Xdd1-GFP)* (F) and *Tg(UAS:Fzd3a*Δ*C-GFP)* (G), throughout r4 blocks FBMN migration out of r4. (H,I) Chimeric embryos with transplant conditions indicated as donor→ host. Cascade blue-dextran marks all donor-derived cells (blue) and *Tg(isl-1:mRFP)* marks all donor-derived FBMNs (magenta). (H) Wild type donor-derived FBMNs migrate normally in a non-transgenic control host. N=37 embryos, 378 FBMNs. (I) Wild type donor-derived FBMNs fail to migrate out of r4 that is expressing *Tg(UAS:Xdd1-GFP)*. N=26 embryos, 190 FBMNs. Inset: same image without the magenta channel showing that donor-derived FBMNs (blue, circled) are not themselves expressing Xdd1-GFP (green). (J) Histograms indicate the percent of donor-derived FBMNs at 48 hpf that failed to migrate (r4), migrated partially (r5) or migrated fully (r6). Each histogram corresponds to the chimeric condition in the image to its left. ***$p<0.0001$ compared to WT → WT control. Significance was determined using a χ^2 test (χ^2=207.8). Scale bar: 50μm. I would like to acknowledge A. Mathewson for images and data in panels C-J.

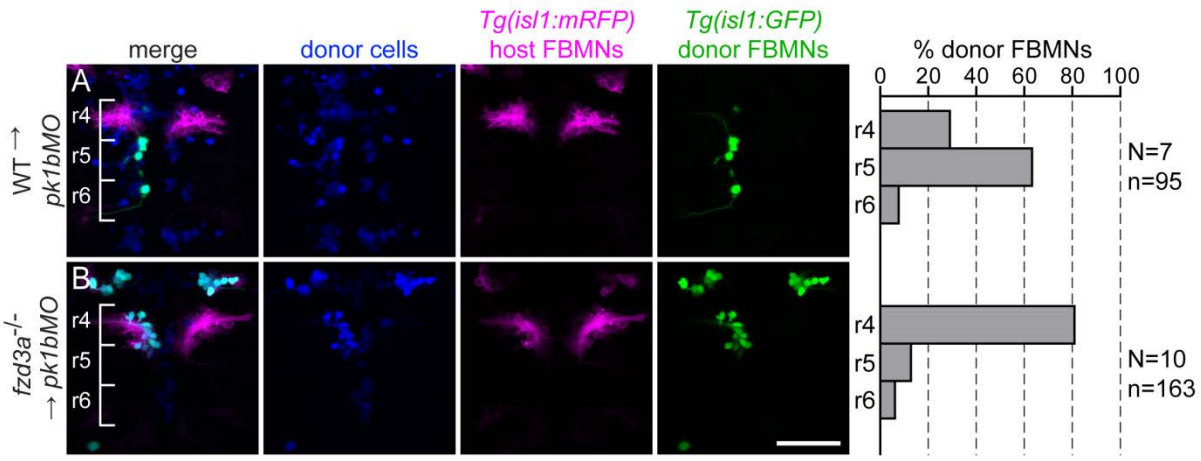

Figure 3: Fzd3a has a cell-autonomous function in FBMN migration.

(A-B) Live confocal images at 48 hpf of chimeric embryos with anterior to the top. Transplant conditions are indicated as donor→host. Pk1bMO host embryos were used because they have normal neuroepithelial planar polarity but unmigrated FBMNs; this prevents donor-derived FBMNs from being carried to r6 by migrating host neurons in a PCP-independent manner. Cascade blue-dextran marks all donor-derived cells (blue), *Tg(isl1:mRFP)* marks host FBMNs (magenta) and *Tg(isl1:GFP)* marks donor-derived FBMNs (green). Histograms on the right indicate the percent of donor-derived FBMNs at 48 hpf that failed to migrate (rhombomere (r)4), partially migrated (r5) or fully migrated (r6). N indicates the number of chimeric embryos and n indicates the number of FBMNs scored in each condition. Brackets indicate rhombomere positon. Scale bar: 50 µm.

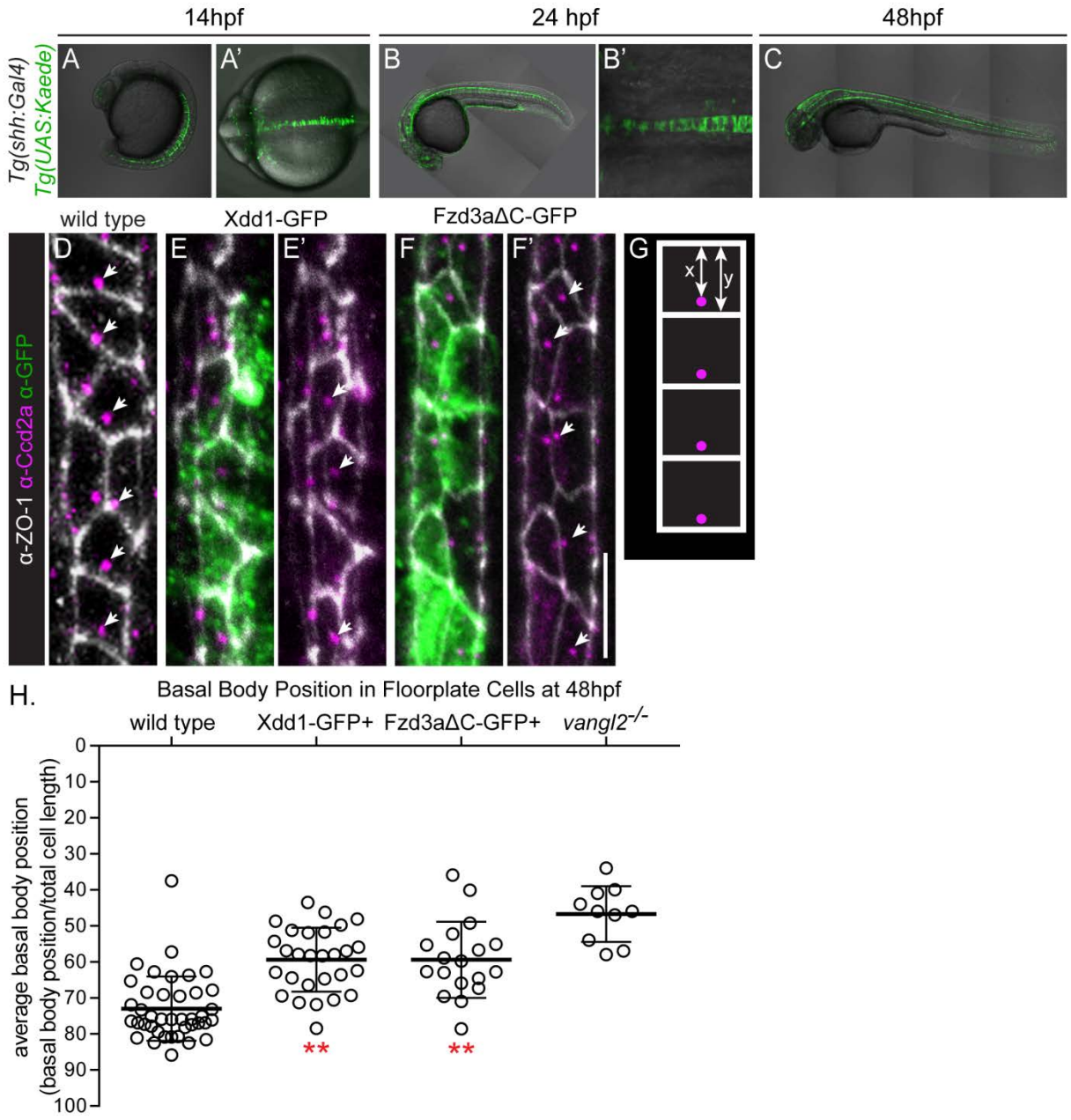

Figure 4: PCP-DN expression in the floorplate disrupts planar polarity.

(A-C) *Tg(shh:Gal4)* driven expression of *Tg(UAS:Kaede)* in the notochord and floorplate of a 14 hpf (A) 24 hpf embryo (B) and a 48 hpf embryo (C). Anterior is to the left. Images are live lateral views in A-C and live dorsal views at the hindbrain level, A',B'. (D-F) Confocal images showing floorplate planar polarity of the anterior spinal cord in 48 hpf zebrafish embryos.

Anterior is to the top. Anti-ZO-1 marks subapical tight junctions (white), anti-Cc2d2a marks the basal bodies of the primary cilia (magenta, arrows), and anti-GFP indicates dominant negative protein expression (green). Scale bar: 10μm. Whereas basal bodies are localized toward the posterior membrane in wild type embryos (D), this polarity is disrupted in floor plate cells expressing Xdd1-GFP (E) or Fzd3aΔC-GFP (F) (arrows in E' and F'). (G) Schematic of the method used to quantify floorplate planar polarity. Total cell length (x) is measured as the distance between the anterior and posterior membranes (white) at the level of the basal body (magenta). Basal body position (y) is measured as the distance between the anterior membrane and the basal body. Cellular planar polarity is quantified as the ratio of x/y. (H) Quantitation of average basal body position in the floor plate of 48hpf embryos. Each data point represents the mean basal body position for all cells quantitated in a single embryo. WT: N=34 embryos, 411 cells; Xdd1-GFP: N=14 embryos, 207 expressing cells; FzdΔC-GFP: N=29 embryos, 484 expressing cells; *vangl*$^{-/-}$: N=10 embryos, 96 cells. Quantitation of floorplate polarity in *vangl2*$^{-/-}$ embryos is included for comparison. Graph represents data as mean ± SD. **p<0.0001 compared to wild-type control. I would like to acknowledge A. Mathewson for the data in this figure.

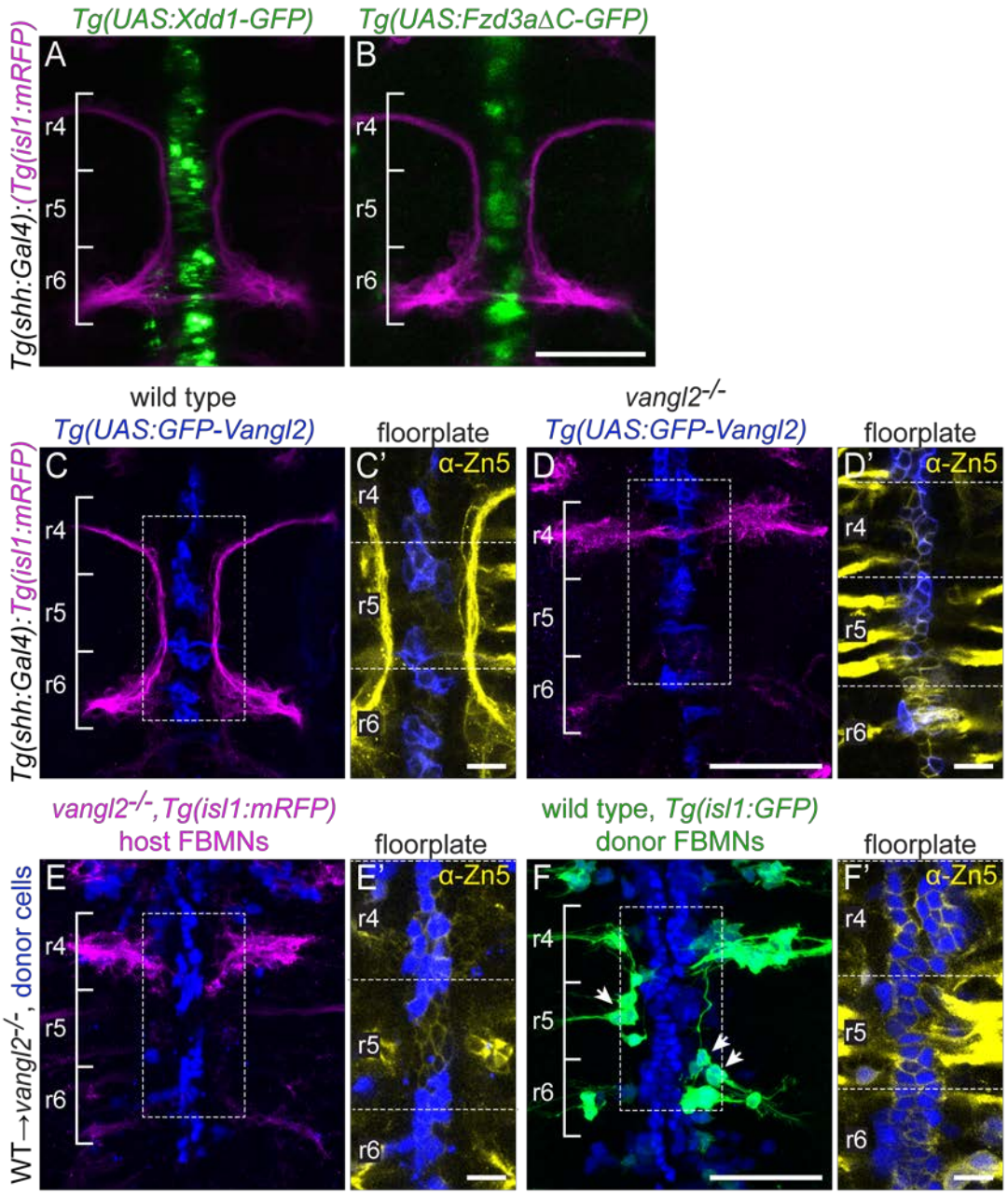

Figure 5: Floorplate PCP can support - but is not sufficient for - FBMN migration.

Confocal images showing dorsal views of 48 hpf hindbrains with anterior to the top. (A,B) *Tg(shh:Gal4)*-driven expression of *Tg(UAS:Xdd1-GFP)* (A) and *Tg(UAS:Fzd3a*Δ*C-GFP)* (B) does not disrupt FBMN (magenta) migration. N= 13 Xdd1-GFP expressing embryos and 26 Fzd3aΔC-GFP expressing embryos. (C,D) *Tg(shh:Gal4)*-driven floorplate expression of GFP-

Vangl2 (blue) in the floorplate of a wild type sibling does not disrupt FBMN migration (magenta) (C) and does not rescue migration in a *vangl2* mutant (D). N=24 *vangl2* mutants with GFP-Vangl2 expression in the r4 floorplate, 14 with 5 or more expressing floorplate cells in r4. (C',D') Boxed regions from panels C and D respectively, showing a single z-plane where GFP-Vangl2 (blue) is expressed broadly in floorplate cells whose membranes are marked with the Zn5 antibody (yellow) (Trevarrow et al., 1990). (E,F) Genetic chimeras. Cascade blue-dextran marks all donor-derived cells (blue), *Tg(isl1:mRFP)* marks host FBMNs (magenta in E) and *Tg(isl1:GFP)* marks wild type donor-derived FBMNs (green in F). (E) The presence of wild type floorplate cells (blue) in a *vangl2* mutant host embryo does not rescue the migration of host FBMNs. N=16 embryos with extensive contribution of WT cells to the floorplate. (F) The presence of wild type floorplate cells (blue) in a *vangl2* mutant can, however, support the migration of co-transplanted wild type donor derived FBMNs (green, arrows). N=8 embryos with migrated donor-derived FBMNs/22 embryos with donor-derived FBMNs; N=76 migrated FBMNs/383 total donor-derived FBMNs. (E',F') Single Z-planes of the boxed regions from panels E and F respectively, show that donor-derived cells (blue) are in the Zn-5-positive floorplate (yellow). Scale bars: 50 µm, 5µm in the insets. I would like to acknowledge A. Mathewson for the images in panels A-B and C. Moens for the images in panels E-F.

Chapter 4: Localization of PCP Proteins in the Migratory Environment and in Migrating FBMNs

The PCP proteins Vangl2, Fzd3a and Pk1b are planar polarized in the migratory environment

Thus far, we have shown that PCP signaling in FBMNs and their immediate neuroepithelial/floorplate r4 environment can drive migration. The localization of core PCP components is known to be crucial for many PCP mediated processes (Goodrich and Strutt, 2011; Wallingford, 2012). Therefore, to better understand how PCP signaling might be used in neuronal migration we asked where PCP proteins localize within FBMNs and in their neuroepithelial microenvironment. Using a polyclonal antibody against zebrafish Vangl2, we observed localization of Vangl2 to cell membranes throughout the hindbrain neuroepithelium (Fig. 6). In the r4 floor plate, we noted an 1.6-fold enrichment of Vangl2 protein at anterior/posterior membranes of floorplate cells compared to their lateral membranes (Fig. 7A,B). Co-staining with ZO1 shows that this staining is sub-apical, at the level of the tight junctions (Fig. 7A'). In order to distinguish anterior from posterior membrane localization we mosaically expressed GFP-Vangl2 in the floorplate so we could visualize Vangl2 localization in isolated floorplate cells. This revealed that Vangl2 is specifically enriched at the anterior subapical membrane (Fig. 7C). Conversely, Fzd3a-GFP is enriched at the posterior membrane (Fig. 7D). These findings for PCP protein localization in the floorplate are consistent both with the requirement for PCP core components in the posterior localization of the floor plate primary cilium (Borovina et al., 2010), and with a conserved deployment of PCP core components in vertebrate and invertebrate epithelia.

Vangl2 localizes to the membrane of FBMNs and is enriched at the tips of retracting Filopodia

We next sought to determine where Vangl2 localizes in migrating FBMNs. Endogenous Vangl2 in FBMN membranes and the membranes of surrounding cells could not be resolved using the anti-Vangl2 antibody and, unlike static floorplate cells, FBMNS are highly dynamic, extending primarily filopodia-like protrusions as they migrate (Mapp et al., 2010; Walsh et al., 2011). Reasoning that Vangl2 localization would be similarly dynamic, we mosaically expressed GFP-Vangl2 in FBMNs and visualized localization using spinning disc time-lapse imaging. We found that GFP-Vangl2 localizes throughout the membrane as well as in putative cytoplasmic vesicles, as is predicted for a transmembrane protein (Fig. 8A). However, in addition to its membrane localization, we observe transient enrichment of GFP-Vangl2 at the tips of a subset filopodia immediately preceding filopodia retraction (Fig. 8A'-C). Before enrichment the mean fluorescent intensity ratio of GFP at the filopodia tip versus the filopodia base is approximately 1 (0.99 ± 0.01), as is the case for mRFP (background membrane marker) (0.92 ± 0.02). During the enrichment event, this ratio for GFP-Vangl2 increased to 1.31 ± 0.05 while the ratio for mRFP remained close to 1 (0.97 ± 0.02) (Fig. 8B). Since the ratio for mRFP remained close to 1, this suggests that the enrichment of GFP-Vangl2 correlates with increased Vangl2 protein levels at filopodia tips and not simply condensation of the membrane due to retraction. This enrichment of GFP-Vangl2 in filopodia never lasted for more than one time-point (images were taken at 30-45 second intervals) and was only detected in a subset of filopodia (N=11/84 filopodia on 8 neurons in 7 embryos); it is likely that due to the transient nature of enrichment events and the constraints of our imaging rate we failed to observe many enrichment events. Importantly, however, the enrichment events we captured invariably preceded filopodial retraction; filopodia never

extended further after an enrichment event (Fig. 8C). Consequently, we infer that Vangl2 may function in FBMN filopodia to signal retraction events.

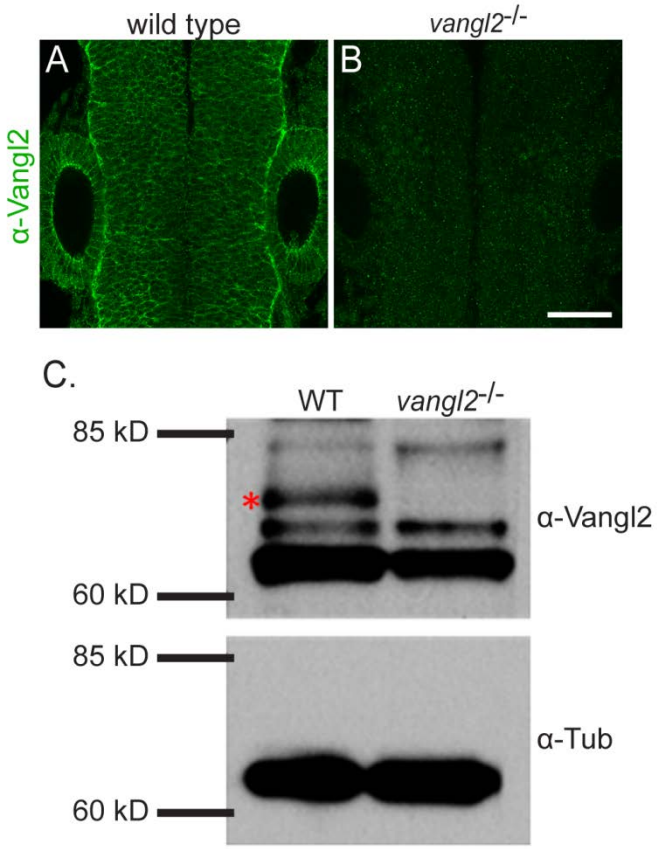

Figure 6: Specificity of the anti-Vangl2 antibody.

(A-B) Dorsal view of wild type (A) and *vangl2* mutant (B) 24 hpf neural tubes immunostained with anti-Vangl2-NT (green). The neuroepithelial membrane staining visible in wild type is absent in the mutant. (C) Western blot analysis of whole embryo lysates with anti-Vangl2 antibody. Anti-alpha-tubulin was used as a loading control. Zebrafish Vangl2 is expected to run at approximately 60kDa. For the anti-Vangl2 blot there is a band that is present in the wild type and absent in the Vangl2 mutant, see asterisk.

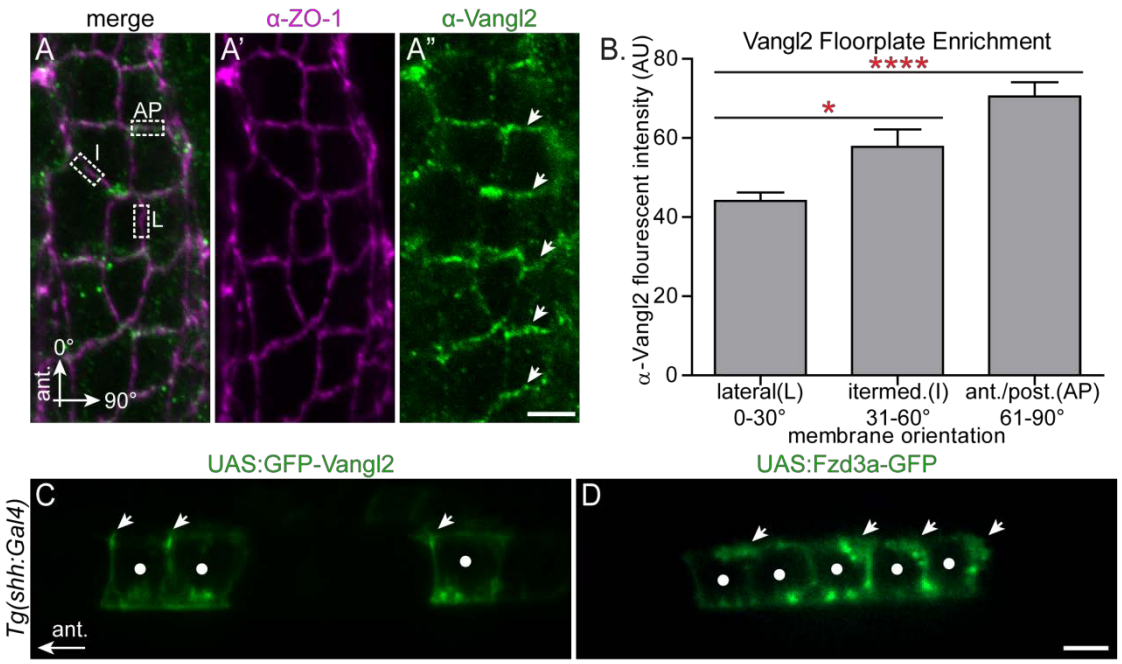

Figure 7: PCP protein localization in the migratory environment. (A-A") Dorsal view with anterior to the top of a 24hpf wild type floorplate at the level of r4 co-immunostained with anti-Vangl2 (green) and anti-ZO-1 (magenta), a marker of apical tight junctions. The boxed regions in A are examples of anterior-posterior (AP) membranes (61-90° from AP axis), intermediate membranes (31-60° from AP axis) and lateral membranes (L) (0-30° from AP axis). Arrows in A" indicate enrichment of anti-Vangl2 labeling at AP membranes. Scale bar: 5 µm. (B) Quantitation of fluorescent intensity of anti-Vangl2 labeling for AP, I and L membranes. N=5 embryos, 192 membranes (57 L, 47 I, 88, AP). (C-D) Live lateral views of 48hpf wild type floorplate cells at the level of the spinal cord with mosaic expression of GFP-Vangl2 (C) and Fzd3a-GFP (D). Anterior is to the left and dorsal/apical is up; white dots indicate the center of each expressing floorplate cell, arrows indicate anterior subapical membrane enrichment of GFP-Vangl2 (C) and posterior subapical enrichment of Fzd3a-GFP (D). Scale bar: 5 µm. Graph represents data as mean ± SEM. *p=0.018, ****p<0.0001; Significance was determined using a paired two-tail t-test with Welch's correction.

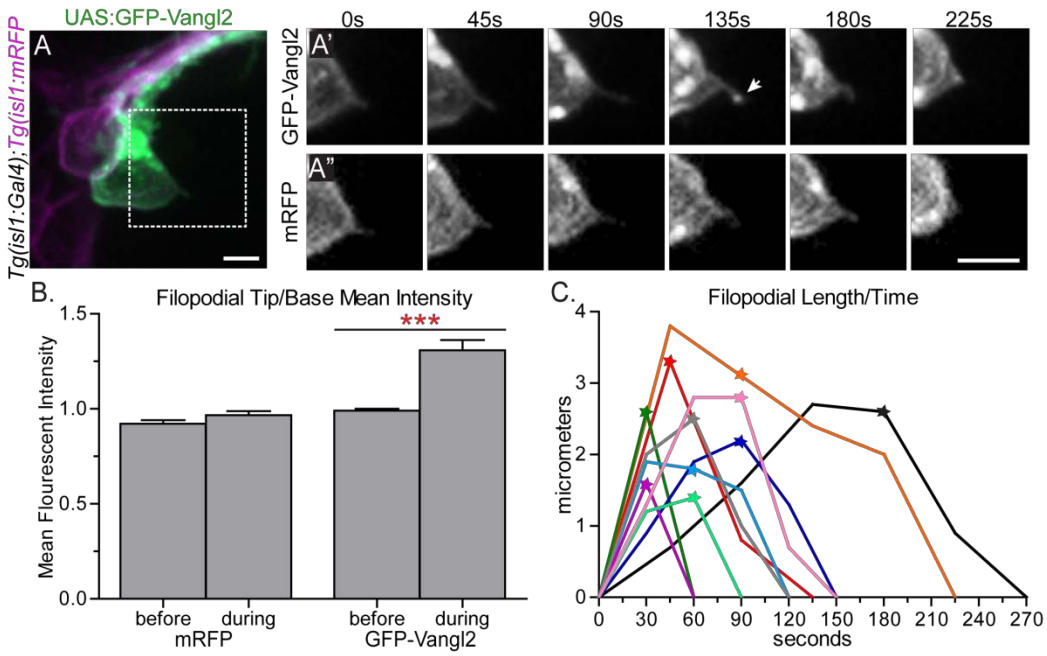

Figure 8: Localization of Vangl2 in FBMNs. (A) Live confocal image of a single GFP-Vangl2 expressing FBMN (green) in a Tg(isl1:mRFP) (magenta) 24 hpf embryo. Scale bar: 10 µm. (A',A") Magnified views of the boxed region in E of the individual channels, GFP-Vangl2 and Tg(isl1:mRFP) respectively, at the time points indicated. The arrow in E' indicates enrichment of GFP-Vangl2 at the filopodial tip. Scale bar: 10 µm. (B) Quantitation of filopodia tip/base mean fluorescent intensity ratio for mRFP and GFP-Vangl2 at the time-point before and during GFP-Vangl2 enrichment. Before enrichment the mean fluorescent intensity ratio of GFP and mRFP at the filopodia tip versus the filopodia base is approximately 1 (N=9 filopodia). During the enrichment event this ratio for GFP-Vangl2 is 1.31 while the ratio remains close to 1 for mRFP (N=12 filopodia). (C) Plot showing the change in filopodial length over time for 10 filopodia. The stars indicate the time-point that GFP-Vangl2 is enriched at each filopodium tip. The black trace corresponds to the filopodium in A',A". Graph represents data as mean ± SEM. ***$p<0.001$. Significance was determined using an unpaired, two-tail t-test with Welch's correction.

Chapter 5: Vangl2 And Fzd3a have Opposing Cell-Autonomous and Non-Cell-Autonomous Roles in Regulating FBMN Filopodial Activity

Vangl2 and Fzd3a function cell-autonomously to regulate FBMN filopodial activity in an antagonistic manner

Our findings that PCP signaling is required within FBMNs for migration, and that Vangl2 localizes transiently to the tips of retracting filopodia, suggested the possibility that PCP signaling influences filopodial dynamics in migrating neurons *in vivo*. In order to determine the cellular basis of FBMN migration defects in PCP mutants, we imaged the protrusive dynamics of single mutant FBMNs at high resolution *in vivo*. Previous studies have described membrane protrusions in fixed or live embryos expressing cytoplasmic GFP or membrane-RFP in bulk FBMNs at low time resolution, however the overlap between FBMNs allows only a subset of protrusions to be visualized and their dynamics could only be inferred from distant time points (Mapp et al., 2010; Wanner and Prince, 2013). To visualize the protrusive activity of single FBMNs at high time resolution, we utilized cell transplantation to generate embryos in which one or a few FBMNs express membrane-localized teal fluorescent protein (*Tg(isl1:mTFP)*), and imaged protrusion dynamics of single FBMNs at 30-second intervals, the shortest interval at which we could acquire comprehensive z-stacks on our instruments. We focused on the function of Vangl2 and Fzd3a, the mutually antagonistic transmembrane core components, whose localized activity is both the hallmark and the driver of classical epithelial planar polarity (Goodrich and Strutt, 2011).

Time-lapse imaging revealed that filopodia are the prevalent protrusion type in FBMNs (Fig. 9A-E). To characterize protrusive membrane dynamics we quantified filopodial lifetime

(number of seconds each filopodium is present during a 15 minute time-lapse period) and filopodial maximum length (the greatest length during the lifetime of filopodia lasting 90 seconds or longer) of *Tg(isl1:mTFP)* FBMNs. Wild type FBMNs generate filopodia with an average lifetime of 224.5 ± 18.66 (SEM) seconds and an average maximum length of 3.6 ± 0.3 μm (Fig.9A,F,G). This filopodial lifetime is comparable to that observed in other vertebrate cells both *in vivo* and in culture (Portera-Cailliau et al., 2003; Lim et al., 2008; Ahmed et al., 2010; Villefranc et al., 2013). When compared to wild type, FBMNs in *vangl2* mutant embryos have much more stable filopodia with a longer average lifetime of 537.3 ± 81.78 seconds (Fig. 9F; p=0.0059). Filopodia of these *vangl2* mutant FBMNs also reach a greater average maximum length of 6.4 ± 1.1 μm (Fig. 9G; p=0.0406). We saw a similar trend when we used microinjection rather than transplantation to mosaically express mTFP in FBMNs in wild type and *vangl2* mutant embryos. These results suggest that Vangl2 is required to destabilize FBMN membrane protrusions.

Since Vangl2 is required within FBMNs *and* their r4 microenvironment for migration, we sought to determine where Vangl2 functions to regulate filopodia dynamics. To determine the cell-autonomous function of Vangl2, we transplanted *vangl2* mutant FBMNs into a wild type host. Donor embryos carried the *Tg(isl1:mTFP)* transgene to visualize FBMNs and contained rhodamine dextran to track other donor-derived cells so we could ensure that donor-derived FBMNs were contacting very few, if any, other donor-derived cells (Fig. 10). We found that *vangl2* mutant FBMNs in a wild type environment have longer, more stable filopodia with a mean lifetime of 432.0 ± 55.65 seconds and a maximum length of 6.8 ± 0.8 μm, similar to *vangl2* mutant FBMNs in a *vangl2* mutant host (Fig. 9B,F,G; p=0.005 and p=0.0078 respectively). To further test if Vangl2 functions cell-autonomously to control FBMN protrusive dynamics, we

mosaically expressed GFP-Vangl2 in wild type FBMNs. FBMNs expressing GFP-Vangl2 in wild type embryos have less stable filopodia compared to wild type FBMNs, with an average lifetime of 123.4 ± 14.27 seconds (N=6 embryos, 7 neurons, 42 filopodia, p=0.0013). Together, these loss- and gain-of-function findings suggest that Vangl2 functions within FBMNs to destabilize filopodia, since filopodia are affected in *vangl2* mutant and GFP-Vangl2-expressing FBMNs regardless of the genotype of cells in their microenvironment.

Fzd3a, like Vangl2, is required cell-autonomously and cell non-autonomously for FBMN migration (Fig. 3, (Wada et al., 2006)). To determine whether Fzd3a has a cell-autonomous role in FBMN protrusive activity, we transplanted *fzd3a* mutant FBMNs into a wild type host. We found that filopodia of *fzd3a* mutant FBMNs are significantly less stable than filopodia of wild type neurons, with a mean lifetime of 163.3 ± 8.006 seconds (Fig. 9C,F; p=0.0092). However, mean maximum filopodia length (3.4 ± 0.4 μm) was not significantly different than that of wild type FBMNs (Fig. 9G). This suggests that Fzd3a normally functions within FBMNs to stabilize filopodia protrusions, consistent with a conserved role for Fzd in actin polymerization (Strutt et al., 1997; Fanto et al., 2000; Winter et al., 2001). Taken together, our results suggest that Vangl2 and Fzd3a function antagonistically within FBMNs to regulate filopodial stability.

The cell-autonomous function of Vangl2 in regulating FBMN dynamics depends on cells in the migratory environment

Given that we observed this cell-autonomous function for Vangl2 and Fzd3a in regulating FBMN membrane protrusions, we asked whether these proteins regulate FBMN protrusive dynamics independently of cells in the migratory environment. To address this question, we analyzed the protrusive dynamics of isolated FBMNs in primary culture. We found that cultured

FBMNs display altered filopodial dynamics compared to FBMNs *in vivo*. Cultured wild type FBMNs have a mean lifetime of 537.5 ± 32.28 seconds (during a 600 second time-lapse) and an average maximum length of 6.0 ± 0.5 μm (Fig. 12). Furthermore, there is no difference in filopodial dynamics between cultured wild type and cultured *vangl2* mutant FBMNs (Fig. 12). This suggested to us that the cell-autonomous functions we observe for Vangl2 and Fzd3a *in vivo* depend on interactions with cells in the migratory environment.

Vangl2 and Fzd3a have opposing cell *non*-autonomous functions in regulating FBMN filopodial activity

Since Fzd3a and Vangl2 are also required non-autonomously for FBMN migration and since FBMN protrusive dynamics depend on cells in the migratory environment, we asked whether cells in the FBMN environment influence FBMN protrusive activity in a PCP-dependent manner. In order to assess the role of Vangl2 in the environment, we imaged protrusion dynamics of wild type FBMNs in a *vangl2* mutant environment. Interestingly we found that wild type FBMNs have less stable filopodia in a *vangl2* mutant environment compared to a wild type environment, with a mean lifetime of 143.3 ± 18.28 seconds (Fig. 9D, 5F; p=0.0056). The decrease in the average filopodia lifetime of wild type FBMNs in a *vangl2* mutant environment is largely due to these neurons having a larger proportion of filopodia present for only one (30 seconds) or two (60 seconds) time-points (Fig. 11). The mean average length however was not different between wild-type FBMNs in a wild type environment and wild type FBMNs in a *vangl2* mutant environment (Fig. 9G) (3.1 ± 0.2 μm).

Removing Fzd3a from the migratory environment had the opposite effect on FBMN filopodia. Wild type neurons in a *fzd3a* mutant environment generate dramatically more stable

filopodia compared to those in a wild type environment, with a mean lifetime of 363.8 ± 51.12 seconds (Fig. 9E, 9F; p=0.0273). Together our results suggest that the core PCP components Vangl2 and Fzd3a antagonize each other's activity to control filopodial dynamics during neuronal migration *in vivo* and they do so by functioning both within FBMNs and in cells in their migratory environment.

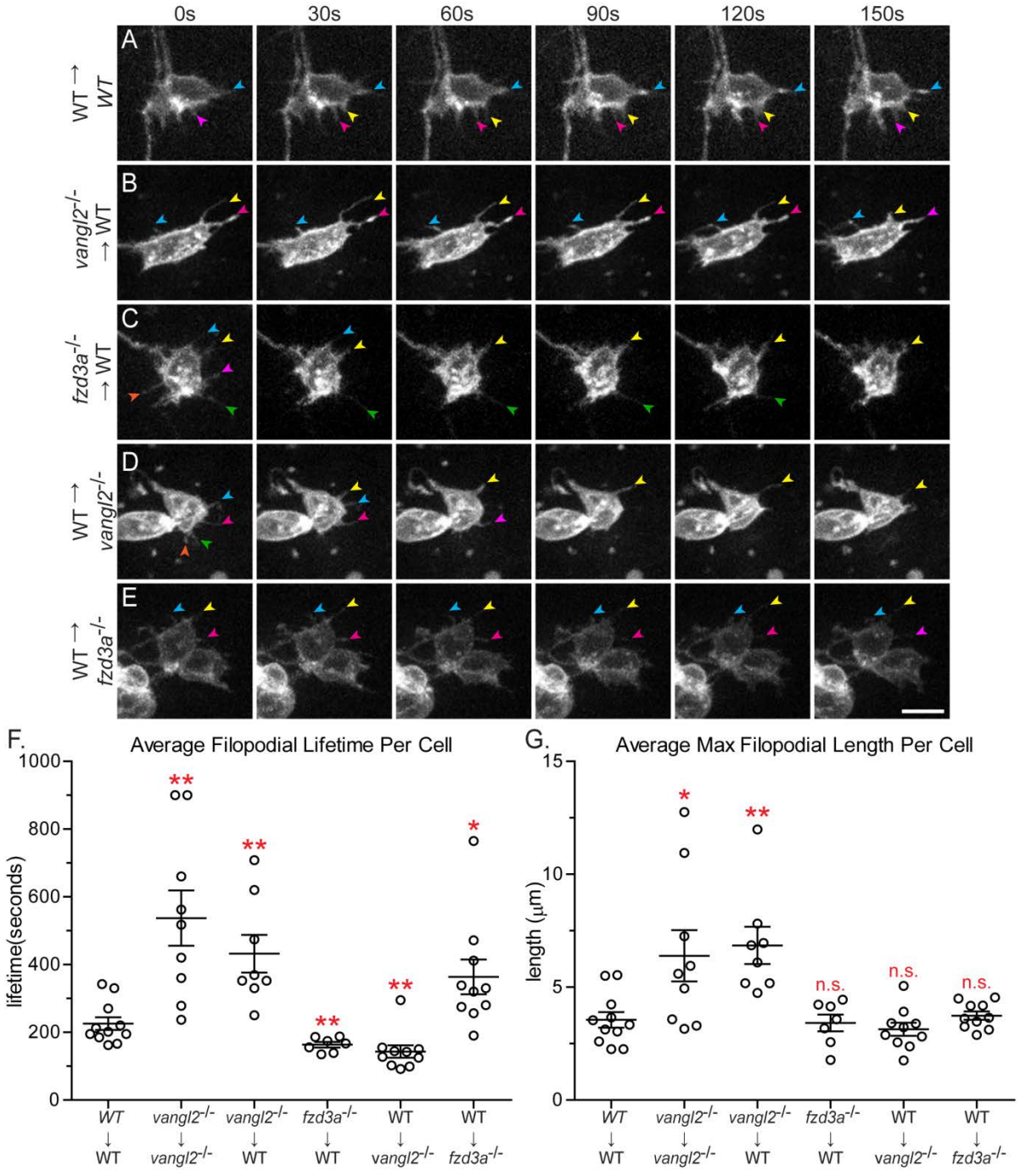

Figure 9: Vangl2 and Fzd3a have opposing cell-autonomous and non-cell-autonomous roles in modulating filopodial dynamics. (A-E) Time-lapse spinning-disc confocal series of donor-derived FBMNs in chimeric embryos at 24-30 hours post-fertilization (hpf). Transplant conditions are indicated on the left as donor→host. Colored arrows indicate individual filopodia

at different time-points. Anterior is to the top and medial is to the right. Scale bar: 5 μm. (F) Quantitation of filopodial lifetime for donor-derived FBMNs. Each data point is an average of all the filopodial lifetimes for one FBMN. (G) Quantitation of the maximum filopodial length for donor-derived FBMNs. Each data point is the average maximum length for all the filopodia of one FBMN. WT→WT: N=6 embryos, 11 neurons (3 in r4, 4 in r5, 4 in r6), 70 filopodia; *vangl2*$^{-/-}$→*vangl2*$^{-/-}$: N=6 embryos, 9 neurons, 43 filopodia; *vangl2*$^{-/-}$→WT: N=6 embryos, 8 neurons, 44 filopodia; *fzd3a*$^{-/-}$→WT: N= 7 embryos, 7 neurons, 73 filopodia; WT→ *vangl2*$^{-/-}$: N= 8 embryos, 10 neurons, 152 filopodia; WT→ *fzd3a*$^{-/-}$: N=6 embryos, 10 neurons, 65 filopodia. Graphs represent data as mean ± SEM. *p<0.05, **p<0.01 compared to WT→WT control; n.s., not significant. Significance was determined using an unpaired, two-tail t-test with Welch's correction.

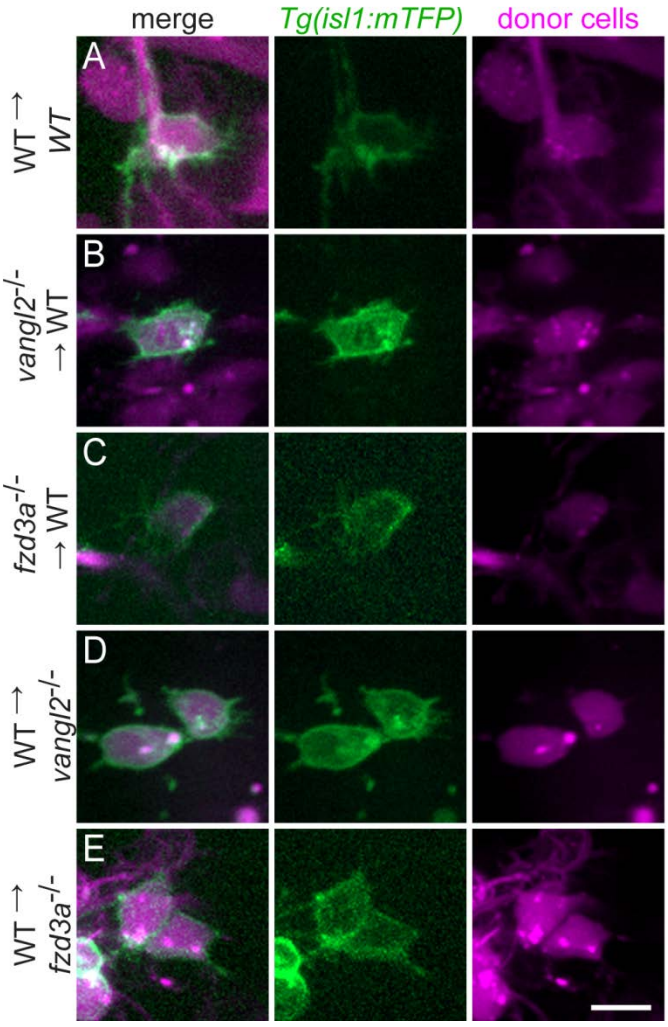

Figure 10: Donor-derived FBMNs used to quantitate filopodial dynamics in chimeric embryos were contacting very few if any, other donor-derived cells. Live confocal images of donor-derived FBMNs (green) and all other nearby donor-derived cells (magenta). Transplant conditions are indicated on as donor→host as in Fig. 5. Rhodamine dextran marks all donor-derived cells (magenta), *Tg(isl1:mTFP)* marks donor-derived FBMNs (green). Anterior is to the top and medial is to the right. Scale bar: 5 μm.

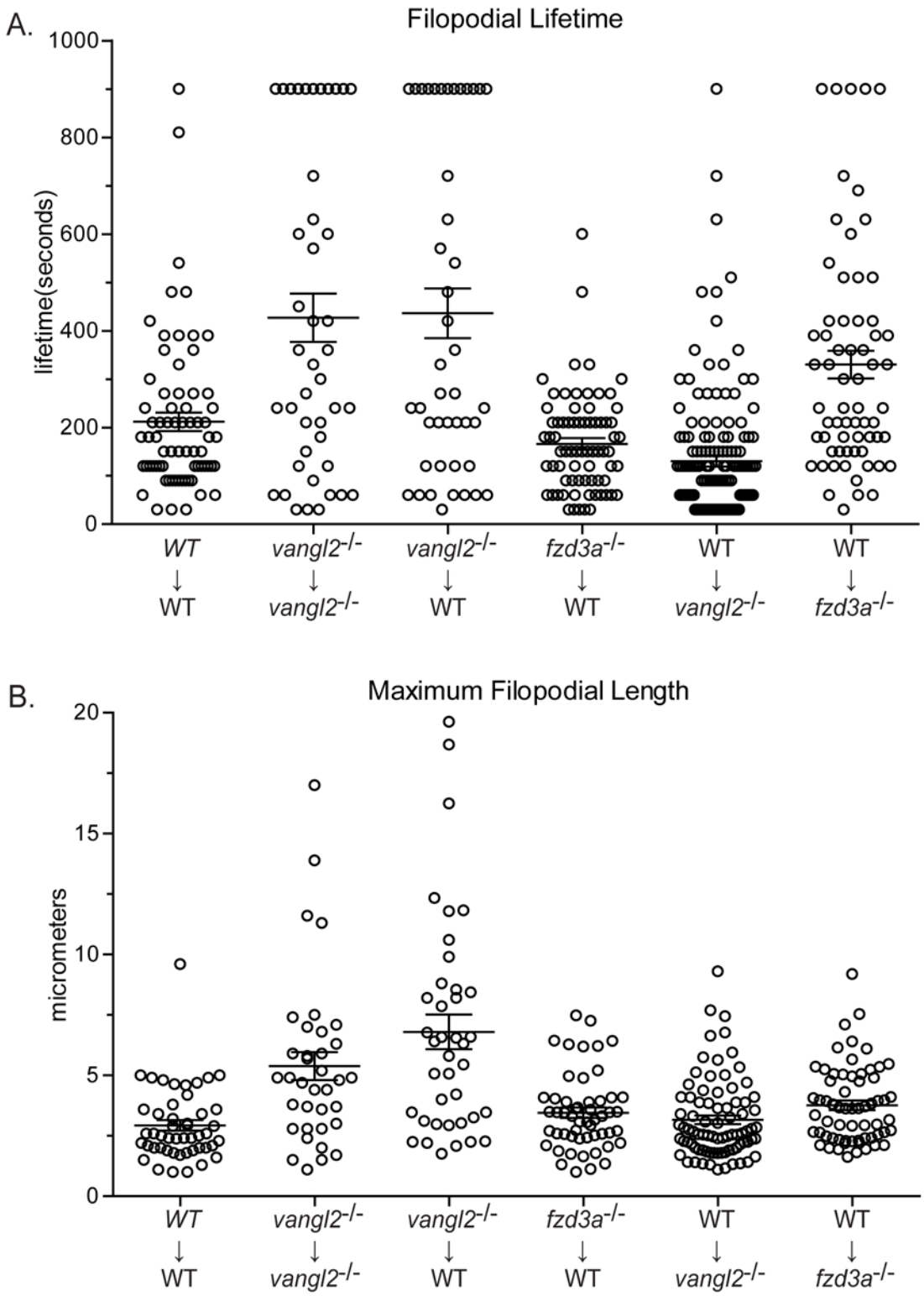

Figure 11: Raw filopodial quantitation data. (A). Quantitation of filopodial lifetime for donor-derived FBMNs. Each data point represents one filopodium. The maximum filopodial lifetime

(900 seconds) corresponds to the full length of the time-lapse. (B). Quantitation of maximum filopodial length for filopodia lasting longer than 90 seconds on donor-derived FBMNs. Each data point represents one filopodium.

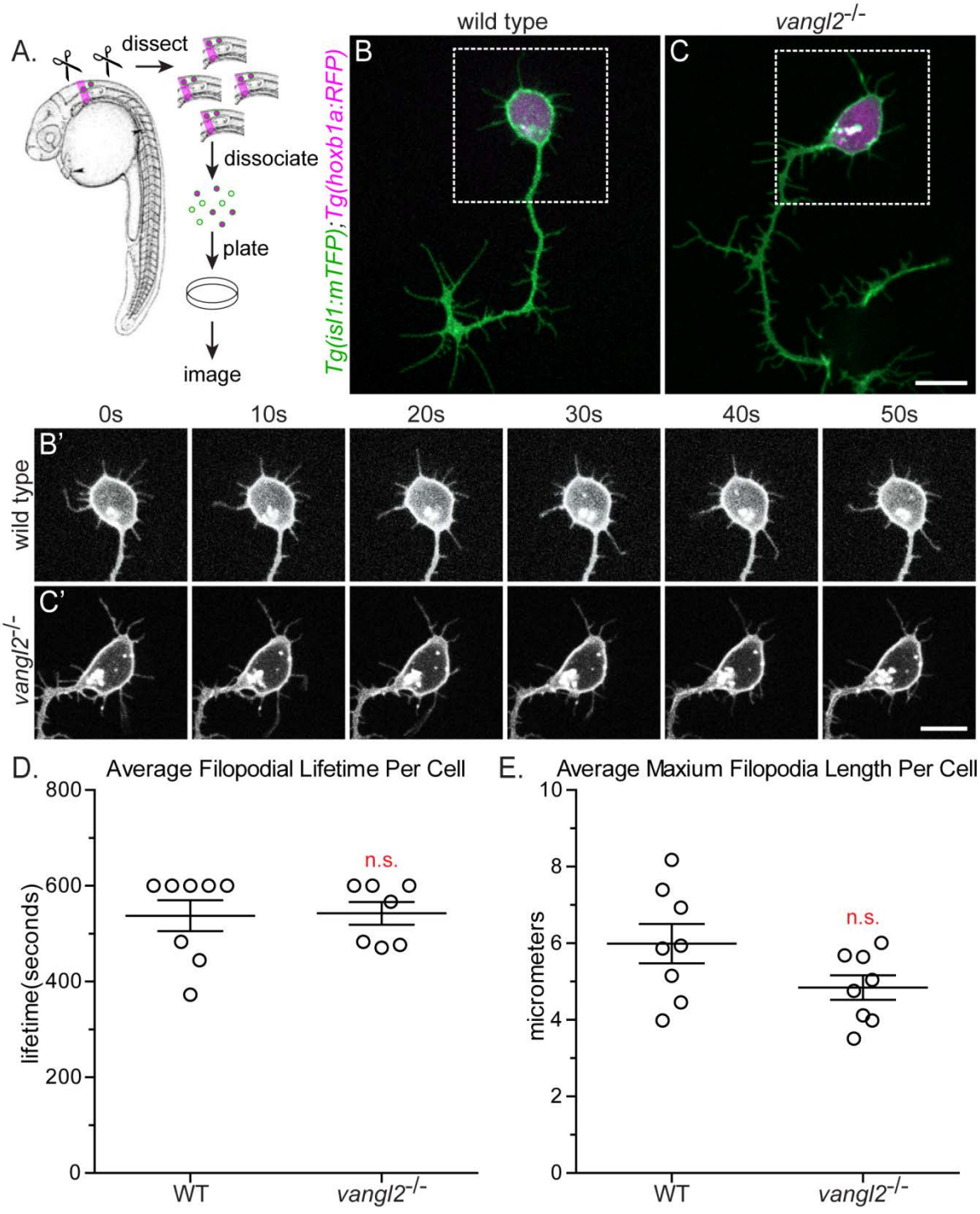

Figure 12: The effect of PCP on protrusion dynamics is dependent on the migratory environment. (A) Method used to isolate and identify FBMNs in primary culture. Embryos used were *Tg(isl1:mTFP);Tg(hoxb1a:RFP)* allowing for the differentiation between FBMNs and other

branchiomotor neurons labeled by *Tg(isl1:mTFP)*. (B,C) Cultured *Tg(isl1:mTFP)*; *Tg(hoxb1a:RFP)* FBMNs from a wild type (B) and a *vangl2* mutant embryo (C). (B',C') Time-lapse spinning-disc confocal series of boxed region from B and C. (D) Quantitation of filopodial lifetime for cultured FBMNs. Each timelapse was 600 seconds total. p=0.9044, n.s. (E) Quantitation of the maximum filopodial length for cultured FBMNs. p=0.0856, n.s. Wild type: N= 8 neurons, 64 filodpodia. *vangl2*$^{-/-}$: N= 8 neurons, 61 filodpodia. Graphs represent data as mean ± SEM. Each data point is the average lifetime (D) or maximum length (E) for all the filopodia of one FBMN. Significance was determined using an unpaired, two-tail t-test with Welch's correction.

CHAPTER 6: Conclusions and Future Directions

A model for the role of PCP signaling in FBMN migration

This study provides new insights into the role of the planar cell polarity pathway in neuronal migration by identifying when and where PCP signaling is required and how it affects the dynamic cell behaviors of migrating neurons *in vivo*. The findings presented here suggest a model for the role of PCP signaling in FBMN migration in which canonical interactions between the transmembrane PCP core components Vangl2 and Fzd3a regulate filopodial dynamics, thereby signaling and/or regulating adhesion for directional migration (Fig. 13). This model for filopodial dynamics is based on genetics and this work does not elucidate the molecular nature of these interactions, which remain controversial even in the context of epithelial polarity (Chen et al., 2008; Wu and Mlodzik, 2008). This model is consistent with 1) a dual cell-autonomous and cell-non-autonomous requirement for PCP signaling and PCP core components, specifically for the transmembrane components Vangl2 and Fzd3a, in FBMNs and their rhombomere 4 environment for migration; (this work, (Jessen et al., 2002; Wada et al., 2006; Walsh et al., 2011)); 2) the cytoskeletal and conserved molecular planar polarization of the r4 neuroepithelial environment including the floorplate (this work, (Borovina et al., 2010)); 3) the ability of the planar polarized floorplate to promote the migration of wild type but not mutant FBMNs; 4) the localization of Vangl2 to retracting FBMN filopodial tips; 5) the antagonistic cell-autonomous roles of Fzd3a and Vangl2 in FBMN filopodial stability and 6) the opposite roles of Fzd3a and Vangl2 in the FBMN environment on FBMN filopodial stability. Whether neuroepithelial planar polarity directs posterior migration or simply enables it, and through what effectors PCP signaling regulates filopodial dynamics in vivo are important questions to be answered in future work.

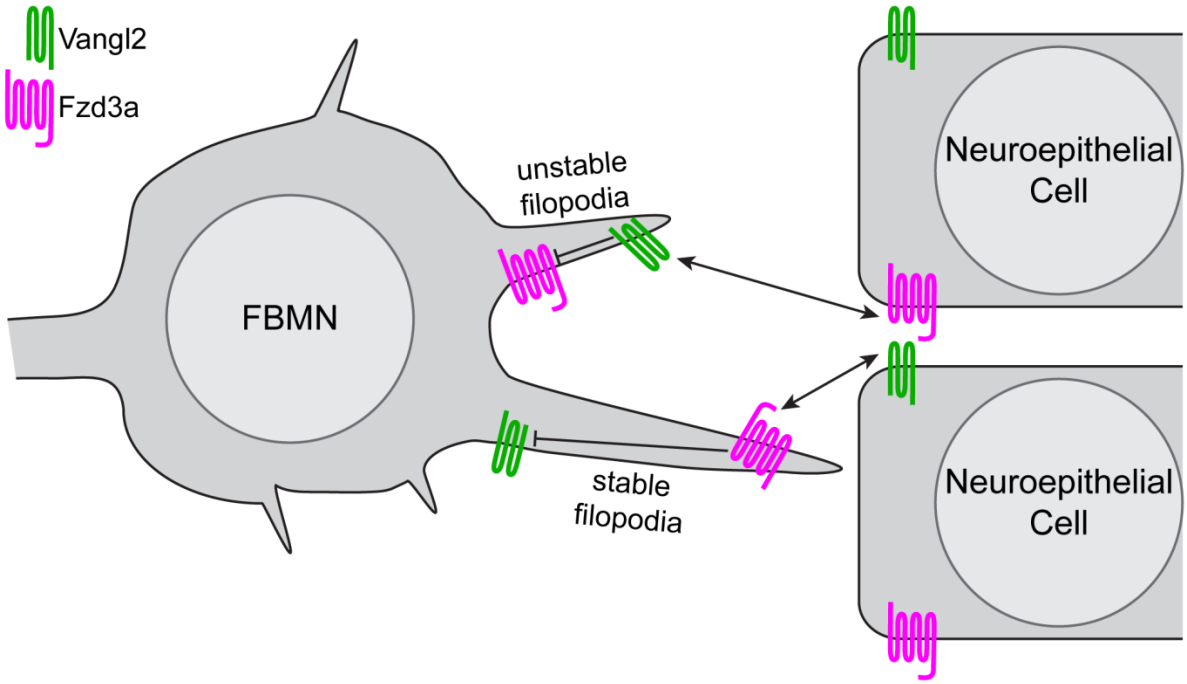

Figure 13: Model of PCP regulation of directed neuron migration. The filopodial dynamics and migratory behaviors of FBMNs observed in genetic chimeras, and the localization of Vangl2 and Fzd3a observed in FBMNs and the cells of their migratory environment, specifically the floorplate, suggest a model in which antagonistic interactions between Vangl2 and Fzd3a mediate the observed effects on FBMN filopodial dynamics and through them, directional neuron migration. Within FBMNs, Vangl2 (green) localizes to filopodial tips and destabilizes them while Fzd3a (magenta) has the opposite, stabilizing effect. In the planar-polarized cells of the migratory environment Vangl2 serves to stabilize filopodia while Fzd3a destabilizes them. In light of the known intracellular and intercellular interactions between Vangl and Fzd that underlie epithelial planar polarization, I hypothesize that interactions between Fzd3a and Vangl2 complexes destabilize one another intracellularly while they promote one another's effects on the actin cytoskeleton when they interact across cell membranes. Whether these interactions provide *directional* cues for migration remains to be discovered.

Mutual Antagonism of Vangl2 and Fzd3a in Migrating Cells

My *in vivo* observations of filopodial dynamics in genetic chimeras demonstrate an antagonistic intracellular relationship between Vangl2 and Fzd3a in migrating FBMNs that regulates the stability of filopodium-like protrusions. While occurring in the context of a highly dynamic structure, this antagonistic relationship of Vangl2 and Fzd3a is reminiscent of the situation in stably polarized epithelia, where mutual intracellular antagonism between Fzd and Vangl complexes sets up polarized actin dynamics within the cell, with Fzd activating actin polymerization distally and Vangl suppressing it proximally (Strutt et al., 1997; Fanto et al., 2000; Winter et al., 2001; Strutt and Warrington, 2008; Yan et al., 2008). This conserved interaction between Fzd promoting and Vangl suppressing actin growth may be common to other migratory cells. In metastatic breast cancer cells induced by stromal Wnt11-containing exosomes, Fzd6 and Vangl2 exhibit mutually exclusive localizations, with Fzd6 on the leading edge of cell protrusions and Vangl2 on non-protrusive cell surfaces, and knock-down of either protein decreases cell motility (Luga et al., 2012). Similarly in migrating leukemia cells, Dvl-3 (part of the Fzd complex) localizes to the leading edge while Vangl2 localizes to the trailing edge (Kaucka et al., 2015). During mesodermal and neuroectodermal convergence, mediolaterally oriented cell surfaces exhibit increased actomyosin contractility (Nishimura et al., 2012; Shindo and Wallingford, 2014) that correlates with the asymmetric localization of PCP components Dvl and Pk (part of the Vangl complex) (Ciruna et al., 2006; Yin et al., 2008), suggestive of a conserved intracellular antagonism of these complexes mediating actin dynamics. In contrast, in commissural growth cones, Vangl2 promotes Fzd3-dependent outgrowth induced by diffusible Wnt5a by antagonizing a non-canonical inhibitory interaction between Dvl1 and Fzd3 identified in that context (Shafer et al., 2011). These examples show that core PCP components localize to

discrete domains of moving cells and I have shown *in vivo* for the first time that this results in opposing effects on protrusion dynamics.

By expressing GFP-Vangl2 specifically in FBMNs I found that Vangl2 localizes to the membrane of FBMNs and is enriched to a subset of retracting filopodia. However, this approach inevitably results in overexpression of the protein of interest, which is a concern because it could cause numerous artifacts such as ectopic sub-cellular localizations and incorrect formation of protein complexes. One way to test if this observed localization of GFP-Vangl2 is accurate and is controlled by a mutual intracellular antagonism between Vangl2 and Fzd3a complex members is to determine if the loss of Fzd3a and of other core PCP components such as Pk1b in FBMNs affects the localization of GFP-Vangl2. It will also be important to determine the localization of other core PCP components in FBMNs to determine if they localize to discrete domains, which would further suggest an intracellular antagonistic relationship between Fzd3a and Vangl2 complexes similar to what is observed in stationary epithelia and a mechanism by which these proteins affect filopodial dynamics in an opposing manner. Attempts to analyze localization of other components using an exogenous approach have proven to be difficult due to expression level issues. This issue could be resolved by knocking in endogenous gene reporters using CRISPR/Cas9 (Auer et al., 2014).

The cell-autonomous opposing functions of Fzd3a and Vangl2 in regulating FBMN filopodia suggests that these proteins have an intracellular antagonistic relationship similar to what is observed in stationary epithelia. However, in this study I did not elucidate the molecular nature of this interaction. Furthermore, although unlikely, it is possible that Vangl2 and Fzd3a are regulating protrusive activity by interacting with independent pathways. If the mechanism by which Vangl2 modulates FBMN filopodial dynamics is strictly via antagonizing the activity of

Fzd3a, a Vangl2-Fzd3a double mutant FBMN should have similar protrusive behavior to that of an Fzd3a mutant. The molecular nature of the relationship between Vangl2 and Fzd3a can be tested by analyzing the requirement of the interactions between Vangl2 and Fzd3a with the cytoplasmic core PCP components Pk and Dsh. These interactions have been shown to require the C-terminal domains of Vangl2 and Fzd3a and are known to be essential for the mutual antagonism of Vang and Fzd in stationary epithelial (Bastock et al., 2003; Jenny et al., 2003; Jenny et al., 2005).

The Role of Filopodia in FBMN Cell Migration

Filopodia are commonly associated with the promotion of directed cell migration, although in some instances, axons and cells can achieve proper targeting and guidance without filopodia (Dwivedy et al., 2007; Phng et al., 2013; Boer et al., 2015). Due to their dynamics and long thin architecture, filopodia are capable of probing a wide area around cells, and they can contain receptors for diverse diffusible and membrane-bound signals and extracellular matrix molecules (Mattila and Lappalainen, 2008). Thus, it is thought that the primary function of filopodia is as “antennae” that cells use to sense their microenvironment to orient directed cell migration (Heckman and Plummer, 2013). Indeed it has been demonstrated that elimination of filopodia in axon growth cones does not impair axon outgrowth, but instead impairs growth cone turning in response to environmental cues (Bentley and Toroian-Raymond, 1986; Chien et al., 1993; Zheng et al., 1996). This sensing role for filopodia has also been demonstrated in cell migration (Boer et al., 2015; Meyen et al., 2015). In addition to a sensing role, filopodia are thought to contribute directly to cell motility, as cells lacking filopodia tend to migrate more slowly due to the absence of filopodial adhesion molecules which could induce traction and also

through force generated by actin streaming in filopodia (Steketee and Tosney, 2002; Galbraith et al., 2007; Phng et al., 2013; Johnson et al., 2015; Meyen et al., 2015). In the context of FBMN migration, filopodia extend in all directions from neurons when they initiate their migration, and I see no bias in the orientation of the filopodia that are affected in PCP mutants. I hypothesize that filopodia act as sensors of asymmetrically localized cell-surface PCP components on the neuroepithelial cells through which they are migrating and that this sensing fine tunes filopodium dynamics such that these filopodia can promote migration by acting as force generators or appropriately sensing other, as-yet unidentified environmental cues.

Although I have shown that PCP signaling modulates the activity of FBMN filopodia, I have not demonstrated that the observed filopodial phenotypes are causative for FBMN migration defects in PCP mutants. One way to address this issue is to disrupt filopodial dynamics independently of PCP signaling and test the migratory outcome of FBMNs. This can be achieved by using a mutational or tissue specific dominant negative approach to disrupt key proteins involved in filopodia formation including: small GTPases such as Cdc42 and Rho, formins, Ena/Vasp proteins and I-Bar domain proteins (Mattila and Lappalainen, 2008).

If FBMNs with disrupted filopodia formation or dynamics fail to migrate, this would suggest that PCP signaling controls migration via regulation of filopodial dynamics. As mentioned above some cells can continue to migrate, albeit it slower and/or in the incorrect direction, in the absence of filopodia. If FBMNs with disrupted filopodia can migrate, this would suggest that additional cell migration mechanisms can compensate for the loss of sensing, adhesion and/or motility and would suggest that PCP signaling also mediates these processes.

Using a mutational approach I tested the requirement of Fascin1a (Fscn1a) and MyosinX-like 1 (Myo10l1) in FBMN migration. Fascins are actin-binding proteins that promote filopodia

formation by bundling F-actin fibers and Fscn1a was recently reported to be required for filopodia formation and migration of a subset of neural crest cells in zebrafish (Hashimoto et al., 2011; Boer et al., 2015). MyosinX is an unconventional myosin that localizes to filopodia and is a potent inducer of filopodia (Kerber and Cheney, 2011). I found that FBMN migration is unperturbed in *fscn1a* maternal zygotic mutants and in *myo10l1*^{fh411} mutants, suggesting that these genes are not required for FBMN migration. However, I have not addressed whether or not FBMN filopodia are disrupted in these mutants. It may be that filopodial dynamics are normal and thus FBMN migration is normal in these mutants. This possibility could be due to genetic redundancy as there are four *fascin* genes and three *myosinX* genes in the zebrafish genome.

I have also tried to disrupt FBMN filopodial dynamics and migration using a dominant negative approach. Ena/VASP proteins localize to the tips of filopodia where they promote actin filament elongation by acting as anti-cappers (Ridley, 2011). I overexpressed the tetramerizeration domain (TD) of VASP, which acts as a dominant negative, in FBMNs (Vasioukhin et al., 2000). I expected to observe short stable filopodia in TD-VASP-GFP expressing neurons due to retention of capping proteins at the growing end of F-actin. However, when I mosaically expressed mouse TD-VASP or zebrafish TD-Vasp-GFP in FBMNs using *isl1*:Gal4 or *hoxb1a*:Gal4 I did not observe a migration block or loss of long dynamic filopodia in expressing FBMNs. I saw a similar outcome when I mosaically expressed FP4-Mito in FBMNs. Ena/VASP protein family members bind FP4 motifs via their EVH1 domains and FP4-Mito acts to mistarget these proteins by sequestering them to the mitochondrion (Bear et al., 2000). These results suggest that Ena/VASP proteins are not required for FBMN migration and filopodial dynamics. However, I was unable to determine if expression of these dominant negatives in FBMNs indeed disrupted Ena/VASP protein function. Further mutational and

dominant negative approaches will be necessary to determine the function of filopodia in FBMN migration.

Factors downstream of PCP signaling that regulate FBMN filopodia dynamics

In other migrating cells, several effectors have been identified as possible links between PCP signaling and cytoskeletal regulation (Habas et al., 2001; Ulmer et al., 2013; Shindo and Wallingford, 2014). However, my work does not elucidate how PCP signaling is transduced to the filopodial actin cytoskeleton in FBMNs. I hypothesize that PCP signals may be transduced to the actin cytoskeleton in FBMNs via the WAVE homology domain (WHD) containing protein Nance-Horan Syndrome-like 1b (Nhsl1b). The WHD domain is found in WAVE (Wiskott-Aldrich syndrome protein family Verprolin-homologous) proteins, which regulate actin polymerization by recruiting the Arp2/3 complex to the membrane (Brooks et al., 2010). Nhsl1b is required cell-autonomously for FBMN migration, physically and genetically interacts with Scrib, is localized to FBMN cell protrusions and its WHD domain is essential for its function in FBMN migration (Walsh et al., 2011). These findings suggest that Nhsl1b functions as a neuron-specific PCP effector that controls migration by modulating actin polymerization at the membrane. Indeed, my preliminary results suggest that Nhsl1b is required for the formation of filopodia in FBMNs. If Nhsl1b is the key effector downstream of PCP signaling that acts to modulate filopodial formation and dynamics, double mutant FBMNs mutant for Nhsl1b and Vangl2 or Fzd3a would have filopodial dynamics similar to Nhsl1b single mutants. In addition, the localization of Nhsl1b to the tips of filopodia would likely be disrupted in PCP mutant FBMNs. This localization and the activity of Nhsl1b may be controlled via its interaction with Scrib. This can be addressed by identifying the domain of Nhsl1b required for its interaction with

Scrib and determining if this domain is required for its function in FBMN migration. Lastly, since Scrib and Vangl2 physically interact, Nhsl1b could be found in a complex with Vangl2 (Montcouquiol et al., 2003; Courbard et al., 2009). Pull down assays could be used to determine if this is the case. This would be interesting because I have demonstrated that Vangl2 functions to destabilize filopodia while Nhsl1b likely functions to stabilize filopodia. It is possible that Nhsl1b is not active when it is in complex with Vangl2 and Scrib. Alternatively, Vangl2 and Nhsl1b may compete for binding with Scrib. Further defining the molecular interaction between Nhsl1b and core PCP proteins and how this affects actin dynamics will be important in determining if Nhsl1b is a PCP effector in migrating FBMNs.

There are a number of other factors that could be acting downstream of PCP signaling in FBMNs to control filopodial dynamics and cell migration. Small GTPases that modulate the actin cytoskeleton, including Rac and Rho, have been shown to act downstream of PCP signaling in *Drosophila* (Strutt et al., 1997; Fanto et al., 2000; Winter et al., 2001; Chung et al., 2007) and downstream of PCP signaling in several vertebrate migration events (Habas et al., 2001; Marlow et al., 2002; Habas et al., 2003; Carmona-Fontaine et al., 2008; Matthews et al., 2008; Nishimura et al., 2012). Using fluorescence resonance energy transfer (FRET) biosensors for Cdc42, Rac and RhoA, it was shown that PCP signaling in neural crest cells likely regulates cellular protrusive activity by activating RhoA (Matthews et al., 2008). Although, challenging it would be interesting to specifically express these biosensors in FBMNs to determine if loss of PCP signaling affects the activity of these proteins. Also dominant negative forms of these proteins could be expressed in FBMNs to determine if this results in similar defects in FBMN migration and filopodial dynamics observed in PCP mutants, which would suggest that PCP signaling in FBMNs is acting through these proteins to promote migration (Feig, 1999). Cdc42 and RhoA can

interact with a number of downstream proteins to modulate actin dynamics involved in filopodia formation (Mattila and Lappalainen, 2008). The effects that loss of PCP signaling and expression of dominant negative forms Cdc42 and RhoA elicit on actin cytoskeletal dynamics could be monitored by expressing Lifeact in FBMNs (Riedl et al., 2008). Activity of these proteins downstream of PCP signaling could also have an effect on actomyosin-dependent contraction (Nishimura et al., 2012; Shindo and Wallingford, 2014), which could occupy a role in FBMN migration, perhaps by driving translocation of the nucleus (Martini and Valdeolmillos, 2010). This could be assayed by staining for activated myosin in FBMNs.

Another pathway that could be functioning downstream of PCP signaling in FBMNs is the c-Jun N-terminal kinase (JNK) pathway, which in addition to its role in regulating transcription is purposed to mediate cell motility (for review, see Xia and Karin, 2004). JNK was shown to be activated downstream of Fz and Dsh to effect planar polarity in the *Drosophila* eye (Boutros et al., 1998; Weber et al., 2000) and JNK was shown to be activated by Wnt5a and required for the guidance of commissural axons (Shafer et al., 2011). Furthermore, JNK signaling in growth cones appears to be controlled by mutual antagonism between Vangl2 and Dvl1 (Shafer et al., 2011). Thus it is possible that JNK signaling downstream of PCP signaling regulates FBMN migration. A common readout of active JNK signaling is phosphorylation of Jun and JNK (Boutros et al., 1998). Immunostaining of phospho-Jun and phospho-JNK could be used to assess whether or not PCP signaling has an effect on JNK signaling in FBMNs. Immunostaining of dissociated FBMNs could be employed to better determine if PCP signaling affects JNK signaling within FBMNs. Alternatively, a JNK activity biosensor could be expressed within FBMNs to specifically assess in *vivo* JNK activity in FBMNs (Fosbrink et al., 2010). Expression of a dominant negative form of JNK within FBMNs or several commercially

available JNK inhibitors could be used to determine whether or not JNK signaling is required for FBMN migration (Liang et al., 2003).

It is also possible that PCP signaling in FBMNs is interacting with more novel effectors to regulate migration (Table 3). Mutational analysis could be used to determine if these proteins and others occupy a role in FBMN migration. Recently our lab developed a rapid reverse genetic screening approach using CRISPR/Cas9 and we demonstrated that this approach can be used to screen for FBMN defects (Shah et al., 2015). This approach can be used to identify novel downstream PCP effectors and factors that act upstream of PCP signaling to control FBMN migration.

In Table 3 Genes highlighted in dark grey have been screened using the CRISPR approach with the help of a summer intern, Sarah Debs, and a lab technician, Arish Shah. These genes did not show FBMN defects in F0 CRISPR injected embryos. However, this could be due to low CRISPR efficiency or genetic redundancy. The genes highlighted in yellow, *lpp* and *sec24b,* displayed convergent extension defects in F0 CRISPR injected animals, suggesting that these genes interact with PCP in this process.

Table 3: Candidate Novel PCP interactors in FBMN migration.

Gene Target	Protein Type/Function	PCP Processes
ankyrin repeat domain 6b (ankrd6b)	*diego* homolog	Gastrulation, heart formation (Moeller et al., 2006)
ADP-ribosylation factor interacting protein 1 (arfp1)	GTP-binding protein.	Vangl2 protein trafficking (Guo et al., 2013)
ATM interactor (*atmin*)	Transcription factor and dynein binding. Genetically interacts with Vangl2.	Oriented cell division (Goggolidou et al., 2014)
calponin2 (*cnn2*)	Actin binding protein.	Neural crest migration (Ulmer et al., 2013)
cilia and flagella associated protein 126 (cfap126)	Uncharacterized protein. Physically interacts with Dvl2.	Ciliogenesis, basal body docking and inner ear hair cell polarization (Gegg et al., 2014)
cofilin1(*cfl1*)	Actin severing protein. Genetically interacts with Vangl2.	Polarization of cilia in the mouse node (Mahaffey et al., 2013)
collagen triple helix repeat containing 1(cthrc1)	Secreted glycoprotein. Genetically interacts with Vangl2. Binds Wnt and Fzd proteins.	Neural tube closure, inner ear hair cell polarization (Yamamoto et al., 2008)
dishvelled associated activator of morphogenesis (*daam1a, daam1b*)	Formin Binds Dvl and promotes filopodia formation.	Gastrulation (Habas et al., 2001)
dishevelled-binding antagonist of beta-catenin 1 (*dact1*)	Scaffolding protein. Physically interacts Vangl2 and Dvl1.	Complexes with Vangl2 to regulate primitive streak formation (Suriben et al., 2009)
GAIP C-terminus interacting protein 1 (gipc1)	Scaffolding protein that regulates cell surface protein trafficking. Physically interacts with Vangl2.	Inner ear hair cell polarization (Giese et al., 2012)
grainy head like 1 (grhl1,grhl2)	Transcription factor. Genetically interacts with Vangl2, Scrib1, Celsr1 and Ptk7.	Epidermal wound closure (Caddy et al., 2010) Neural tube closure (Ting et al., 2003)
inversin	diego homolog	Left-right patterning, Kidney development, Gastrulation (Simons et al., 2005)
inturned	PCP effector	Ciliogenesis, neural tube closure (Park et al., 2006; Heydeck and Liu, 2011)
LIM domain containing preferred translocation partner in lipoma (*lpp*)	Cell adhesion and motility. Genetically interacts with *vangl2* and *scrib*.	Gastrulation (Vervenne et al., 2008)
low density lipoprotein receptor-related protein 5/6 (lrp5/6)	Receptor-mediated endocytosis of lipoproteins and protein ligands. Wnt co-receptor.	Gastrulation (Tahinci et al., 2007)
melanoma cell adhesion molecule (mcam)	Cell adhesion molecule. Binds Wnt5a.	Gastrulation (Ye et al., 2013)
membrane-associated guanylate kinase inverted 2 (magi2)	Scaffold protein. Regulates PTEN activity. Physically interacts with Vangl2.	Podocyte polarity and migration (Babayeva et al., 2011)
membrane type-1 matrix metalloproteinase (mmp14a)	Extracellular matrix breakdown. Levels are regulated by Vangl2.	Gastrulation (Williams et al., 2012)
myosinVI (*myo6a, myo6b*)	Moves toward the minus end of actin filaments and functions in vesicular membrane trafficking and cell migration. Part of the Gipc1-Vangl2 complex.	Inner ear hair cell polarization (Giese et al., 2012)
nitric oxide synthase 1 adaptor protein (*nos1ap*)	Binds nNOS. Complexes with Vangl1 and Scrib and colocalizes with Vangl1 and Scrib in cell protrusions.	Breast cancer cell migration (Anastas et al., 2012)
protein tyrosine kinase 7 (*ptk7*)	Transmembrane tyrosine kinase receptor	Inner ear development, Neural tube closure (Lu et al., 2004; Lee et al., 2012) NC migration (Shnitsar and Borchers, 2008)
rho-associate kinase 2 (Rock2)	Phosphorylates myosin to stimulate actomyosin contractility. Activated downstream of Ptk7.	Inner ear hair cell polarization (Andreeva et al., 2014)
RPGR-interacting protein 1 (*rpgrip1, rpgrip1l*)	Ciliopathy protein. Stabilizes Dsh in the zebrafish floorplate.	Floor plate planar polarization (Mahuzier et al., 2012)
sec14 and sprectrin domains 1 (sestd1)	Docking protein that binds phospholipids. Physically interacts with Vangl2 and Dact1.	Complexes with Vangl2 to regulate primitive streak formation (Suriben et al., 2009)
receptor tyrosine kinase-like orphan receptor 2 (ror2)	Single pass transmembrane with tyrosine kinase domain	Limb bud elongation (binds Wnt5a) (Gao et al., 2011)
sec24b	Vesicle trafficking. Part of COPII coat protein complex.	Inner ear development, Neural tube closure (Merte et al., 2010; Wansleeben et al., 2010)
septin/sept7	Filament forming protein. Interacts with Dvl1 to regulate actomyosin mediated cell cortex tension.	Gastrulation (Shindo and Wallingford, 2014)
testin (*tes*)	LIM domain containing scaffolding protein. Physically and genetically interacts with Vangl2	Inner ear hair cell polarization (Ren et al., 2013)
wilms tumor 1 interacting protein (wtip)	Member of the zyxin family. Genetically interacts with Vangl2.	Spindle orientation (Bubenshchikova et al., 2012)

Fzd3a and Vangl2 Function in the Migratory Environment

A more surprising finding than opposing cell autonomous roles for Fzd3a and Vangl2 in FBMN filopodial dynamics and migration is that the same PCP components function in the FBMN environment to influence filopodial dynamics but in the opposite way: Fzd3a in the environment destabilizes filopodia while Vangl2 in the environment stabilizes them. These non-autonomous functions for Fzd3a and Vangl2 in filopodial dynamics correlate with their non-autonomous requirement for FBMN migration (Jessen et al., 2002; Wada et al., 2006). Again, this is reminiscent of classical planar-polarity, where localized Fzd activity depends on the presence of Vangl in adjacent cells in the epithelium and vice versa; this is the mechanism by which PCP is coordinated across an epithelium (Vinson and Adler, 1987; Taylor et al., 1998; Strutt and Strutt, 2007; Wu and Mlodzik, 2008; Chin and Mlodzik, 2013). One intriguing hypothesis is that the cell-autonomous activities of Fzd3a and Vangl2 are activated in different filopodia when they contact Vangl2 and Fzd3a domains of neuroepithelial cells in the r4 environment (Fig. 13), with consequences on the actin dynamics regulating filopodial stability, leading to changes in signaling and/or adhesion. In this study I have shown that Vangl2 and Fzd3a exhibit planar polarized localization in the floorplate, and neuroepithelial progenitor cells are likewise planar polarized based on GFP-Pk localization (Ciruna et al., 2006). In PCP mutants, this polarized information is absent and/or cannot be correctly interpreted by filopodia resulting in a failure of directional cell migration. The cell-autonomous filopodial phenotypes appear to be dominant, since in constitutive mutants filopodia have the cell-autonomous phenotype (long and stable in Vangl2 mutants; unstable in Fzd3a mutants), which could explain how the cell-autonomous and non-cell-autonomous influence of Vangl2 and Fzd3a on filopodial dynamics do not simply cancel one another out. Together my findings suggest that conserved

intracellular and intercellular interactions between PCP core components can have divergent effects on actin dynamics and consequently on cell behaviors.

Activation of Fzd3a and Vangl2 in different FBMN filopodia upon contact with Vangl2 and Fzd3a domains of neuroepithelial cells in the migratory environment could occur via a direct or an indirect mechanism. A direct mechanism would necessitate an Fzd3a-Vangl2 interaction between dynamic FBMN membranes and neuroepithelial cell membranes in which these proteins recruit and stabilize one another. This type of interaction between Fzd3a and Vangl2 could be inferred by determining how the loss or overexpression of Fzd3a and Vangl2 in the migratory environment affects localization of these proteins in FBMNs. If the Fzd3a-Vangl2 interaction is similar to that observed in stationary epithelia, loss of Fzd3a in the migratory environment would lead to excess Vangl2 activity in the environment and thus increased stabilization or recruitment of Fzd3a to the membranes and filopodia of FBMNs while loss of Vangl2 in the migratory environment would lead to excess Fzd3a activity in the environment and thus increased stabilization or recruitment of Vangl2 to the membranes and filopodia of FBMNs. On the other hand, overexpression of Fzd3a in the environment would result in stabilization of Vangl2 in filopodia and thus less stable filopodia, while overexpression of Vangl2 in the environment would result in stabilization of Fzd3a in filopodia and thus more stable filopodia. These approaches, however, do not rule out disruption of PCP signaling in the migratory environment due to overexpression of PCP components as being an indirect cause of altered protein stability and filopodia dynamics. This could be addressed by specifically disrupting domains shown to be required for the intercellular Fzd-Vang interaction. It has been demonstrated that the extracellular cysteine rich domain (CRD) of Fzd is necessary and sufficient to interact with the extracellular loops of Vangl2 (Wu and Mlodzik, 2008). In the fly wing this domain is required

for PCP establishment and importantly it is required for non-cell-autonomous signaling by Fzd (Boutros et al., 2000; Chen et al., 2004; Wu and Mlodzik, 2008). In a gain-of-function assay FzdΔCRD was demonstrated to localize to the membrane were it recruits Dsh like wild type Fzd, but unlike overexpression of wild type Fzd it did not disrupt wing hair polarity in neighboring cells (Wu and Mlodzik, 2008). Therefore, the non-cell-autonomous function of Fzd3a can be directly tested by expressing Fzd3aΔCRD in the migratory environment. If the non-cell-autonomous function of Fzd3a requires an intercellular interaction with Vangl2, expression of Fzd3aΔCRD would not alter stability of Vangl2 in FBMN filopodia or filopodial dynamics. The reciprocal of this experiment could be performed by overexpressing a form of Vangl2 in the migratory environment that carries point mutations in its extracellular loops which abolish its interaction with Fzd3a (Wu and Mlodzik, 2008).

It has been reported that the stabilizing intercellular interaction between Fzd and Vangl membranes is not due to direct binding of Fzd and Vangl but instead is mediated through the atypical cadherin Fmi, the homolog of Celsr which localizes to both proximal and distal sides of cells and interacts across membranes (Chen et al., 2008). This study proposed that Fmi has two functional forms, one which physically interacts with Fzd and one that physically interacts with Vang, and that these two forms preferentially interact with one another to favor the asymmetric association of Fzd membranes with Vang membranes. If the non-cell-autonomous effects of Fzd3a and Vangl2 on FBMN filopodia dynamics are similarly mediated through Celsr, the opposite effects resulting from Fzd3a and Vangl2 loss in the migratory environment should be abolished in the absence of Celsr function in the migratory environment or in FBMNs.

While the similar effects on filopodial dynamics when Vangl2 is depleted from FBMNS and when Fzd3a is depleted from their environment suggest that the two proteins are working

together, environmental PCP may also influence filopodia dynamics of FBMNs through an indirect mechanism. For instance, core PCP proteins have been implicated in the trafficking and regulation of membrane levels of cadherins in fly and in vertebrate epithelial cells (Classen et al., 2005; Warrington et al., 2013; Nagaoka et al., 2014). Therefore, Vangl2 and Fzd3a in the migratory environment may modulate FBMN filopodia dynamics by regulating N-cadherin levels at the surface of neuroepithelial cells. Another potential mechanism by which PCP in the migratory environment may regulate FBMN filopodial dynamics is through regulation of membrane type-1 matrix metalloproteinase (MMP14), which are known to degrade extracellular matrix proteins. During zebrafish gastrulation, an increase in Mmp14 activity was observed in *vangl2* mutant embryos (Williams et al., 2012). Thus, the decreased FBMN filopodial stability observed when Vangl2 is absent in the migratory environment could be due to decreased extracellular matrix. These hypotheses can be tested by determining if loss of Vangl2 or Fzd3a in the migratory environment alters the levels of these proteins and by determining whether varying levels of these proteins independent of PCP signaling has the predicted effects on FBMN filopodial dynamics.

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