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Planar Cell Polarity and Neurodevelopment

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PLANAR CELL POLARITY IN NEURODEVELOPMENT

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List of Abbreviations

AB	Apicobasal
A-P	Anterior-posterior
CE	Convergent extension
Celsr, CELSR	Cadherin EGF LAG seven-pass G-type receptor
CiD	Circumferential descending
CoBL	Commissural bifurcating longitudinal
CoLA	Commissural longitudinal ascending
CoLo	Commissural local
CoPA	Commissural primary ascending
CoSA	Commissural secondary ascending
D-V	Dorsal-ventral
dcc	Deleted in colorectal cancer
Dgo	Diego
DLF	Dorsal longitudinal fasciculus
Dlg	Disc large
Ds	Dachsous
Dsh, DVL	Dishevelled
FA	Focal adhesions
FBMN	Facial branchiomotor neuron
Fmi	Flamingo
Fj	Four-jointed

Ft	Fat
Fzd, FZD	Frizzled
Gpc4	Glypican 4
GFP	Green fluorescent protein
GUK	Guanylate kinases
HRP	Horseradish peroxidase
IC	Ipsilaterally projecting interneurons
JNK	c-Jun-N-terminal kinase
LAP	LRR and PDZ
Lgl	Lethal giant larvae
LRR	Leucine rich repeats
MIP	Maximum intensity projection
ML	Mediolateral
MCoD	Multipolar commissural descending
MZ	Maternal zygotic
NTD	Neural tube defect
nTSGs	Neoplastic tumor suppressor genes
PCP	Planar cell polarity
PDZ	Post-synaptic density, Dlg1, zonula-occludens-1
Pk	Prickle
Ptk7	Protein tyrosine kinase 7
r4/r6	Rhombomere 4/rhombomere 6

Scrib, SCRIB1	Scribble
SEM	Standard error of the mean
TFP	Teal fluorescent protein
UCoD	Unipolar commissural descending
Vang, Vangl, VANGL	Van Gogh, Van gogh like

Abstract

PLANAR CELL POLARITY IN NEURODEVELOPMENT

By Simon D Sun, B.S.

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

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Planar cell polarity (PCP) is a developmental signaling mechanism that establishes a polarity within the plane of an epithelium. PCP has been shown to play a role in guiding numerous neurodevelopmental processes such as convergent extension, neuron migration, and axon pathfinding. Certain commissural neurons in the dorsal spinal cord make a series of guidance decisions en route to the brain: first, a ventral projection along the D-V axis, followed by a midline crossing, and after exiting the floorplate, a dorso-anterior turn along the A-P axis. Here, we provide *in vivo* evidence that the axons of the Commissural Primary Ascending (CoPAs) neurons in zebrafish require the PCP genes *fzd3a, vangl2,* and *scribble* for rostral pathfinding both before and after crossing the midline. Dorsoventral guidance of CoPA axons is unaltered in *fzd3a, vangl2,* and *scribble* mutants, suggesting that the PCP signaling pathway only

controls A-P guidance of CoPAs. Our results have provided evidence for two potential non-mutually exclusive models: (i) A-P axon guidance is achieved by cell-autonomous Wnt-Frizzled signaling or that (ii) A-P axon guidance is achieved by non-cell-autonomous PCP signaling in the neuroepithelial environment. The single-cell nature of the CoPA axon system allows for simple genetic manipulation and visualization, which will potentially elucidate the validity of either model.

Scribble (Scrib), a member of the LAP family, plays a critical role in establishing and regulating cell polarization in epithelia and during cell migration. In zebrafish, Scrib mutants have defects in convergent extension (CE) cell movements and facial branchiomotor neuron (FBMN) migration. Despite our understanding of Scrib's genetic role in neurodevelopment, little is known about the subcellular localization of endogenous Scrib *in vivo* during CE and FBMN migration. We have generated a monoclonal antibody against the C-terminus of zebrafish Scrib and have shown that this antibody is specific against endogenous Scrib in both western blot and immunocytochemical applications. Confocal microscopy of Scrib immunocytochemistry shows that at various developmental stages, Scrib distinctly localizes to basolateral membranes of non polarized epithelium, to the membrane in mesodermal cells undergoing CE, and to the membrane of migrating FBMNs. Furthermore, the distribution of Scrib puncta along membranes of FBMN-FBMN contact is significantly altered in the PCP mutant *pk1b*. Further application of our newly generated Scrib antibody will potentially lead to new insight on Scrib's role in neurodevelop-ment.

Chapter I: Introduction

Planar cell polarity (PCP), which comprises a subset of non-canonical Wnt signaling, is a developmental signaling mechanism that coordinates the polarity and organization of structures within the plane of an epithelium. PCP was initially described in the wing of *Drosophila melanogaster*, in which each epithelial cell possesses an actin-based hair called a trichome that is asymmetrically localized to the distal side of the cell (Vinson & Adler, 1987). Subsequent studies have identified several "core"-PCP genes that are required for the orientation of trichomes within each wing epithelial cell. These include the seven-pass transmembrane protein Frizzled (Fzd), the atypical seven-pass transmembrane cadherin Flamingo (Fmi), the four-pass transmembrane protein Van Gogh (Vang), and the cytosolic adaptor proteins Dishevelled (Dsh, DVL), Prickle (Pk), and Diego (Dgo). The asymmetric distribution and stabilization of these PCP proteins is what establishes the polarity of these cells, with Fzd, Dsh, and Dgo accumulating at the distal apical junction, while Vang and Prk accumulate at the proximal side. Fmi is the only core PCP protein that is localized to both distal and proximal apical junctions. Current evidence indicates that distal components and proximal components mutually exclude each other to opposite sides of the cell. For instance, Vang and Pk both bind Dsh and inhibit its membrane recruitment and function. In addition, the generation of intracellular asymmetry can be mediated both by the

action of ubiquitin ligases that locally target PCP proteins for degradation (Narimatsu et al., 2009) and polarized recycling and trafficking of Fzd and Fmi to the distal side of cells (Das, Jenny, Klein, Eaton, & Mlodzik, 2004; Giese et al., 2012; Y. Guo, Zanetti, & Schekman, 2013; Shimada, Yonemura, Ohkura, Strutt, & Uemura, 2006; H. Strutt & Strutt, 2005; 2008; Tree, Ma, & Axelrod, 2002). This asymmetrical localization of these two multi protein complexes generates planar polarity signals that are translated into alignment of cells and cellular appendages, such as the distally placed *Drosophila* actin-based hair, with respect to an anatomical axis (figure S1a). A loss of any one of these components results in the loss of the asymmetric localization of the other core proteins (D. Strutt, 2003) and consequently leads to defects in planar polarity, such as the loss of epithelial polarity and hair directionality.

In addition to the core set of PCP genes, there are many other genes that play a role in PCP signaling. Upstream of PCP are global factors, non cell-autonomous signals that coordinate planar polarity across an entire tissue via activity gradients. In the example of the fly wing, these include the atypical cadherins Dachsous (Ds) and Fat (Ft) and the typical II transmembrane protein Four-jointed (Fj), the details of which are beyond the scope of this study, but are reviewed in their context to PCP in (H. Strutt & Strutt, 2005) and in (Tissir & Goffinet, 2013). Downstream, there is a growing list of PCP effectors that serve to translate the established polarity of the core proteins to tissue specific cellular mechanisms (Park, Mitchell, Abitua, Kintner, & Wallingford, 2008; Turner & Adler, 1998; Vladar, Bayly, Sangoram, Scott, & Axelrod, 2012). Some PCP effectors, such as DAAM1 and DAAM2, are formins that interact with Dsh to signal c-Jun-N-terminal kinase (JNK), RhoA, and Rac1, which all play a role in actin bundle elongation and control cytoskeletal dynamics (Habas, Kato, & He, 2001; Liu et al.,

2008). The resulting model describes a global cue, initiated across a developing embryo, that instructs cells within a tissue to establish intracellular polarity through the core PCP components, which is subsequently communicated to PCP-dependent cellular machinery by tissue-specific effectors, resulting in the appropriate orientation and alignment of cellular morphology and architecture (figure S1b).

In addition to establishing intracellular polarity, there has been substantial evidence that these intracellular cues are also communicated to neighboring cells via interactions between the membrane-bound proteins Fzd and Vang. Clones of cells mutant for *fzd* (Gubb & García-Bellido, 1982) or *vang* (Casal, Lawrence, & Struhl, 2006; Taylor, Abramova, Charlton, & Adler, 1998) in wild type tissue results in disruption of the planar polarity in neighboring cells, a process called *domineering non-autonomy*. Specifically, Fzd in the distal side of one epithelial cell (Vinson & Adler, 1987) can stabilize Vang proteins in the proximal side of the neighboring cell (Taylor et al., 1998). The process requires the presence of the atypical cadherin Fmi. Several models suggest that a Fzd-Fmi complex on the distal side of epithelial cells can physically bind and stabilize a Vang-Fmi complex on the adjoining membrane of a neighboring cell (Bastock, Strutt, & Strutt, 2003; Chen et al., 2008; Das, Reynolds-Kenneally, & Mlodzik, 2002; Devenport & Fuchs, 2008; Shimada, Usui, Yanagawa, Takeichi, & Uemura, 2001; Usui et al., 1999). In this way, the planar polarity of an individual epithelial cell can be propagated to the adjacent cells, resulting in the common alignment of neighboring epithelial cells. Indeed, planar cell polarity should be thought of as a form of cell-to-cell communication of polarity information that can coordinate the global alignment of all cells within a tissue.

PCP components and processes in vertebrates

The core PCP proteins described in *Drosophila* have been evolutionarily conserved in vertebrates and analysis of mouse mutants with mutation in the core PCP genes has identified vertebrate planar polarity processes. This analysis has also uncovered additional proteins not found in *Drosophila* that regulate PCP related processes in vertebrate development. Vertebrates possess at least one orthologue of the core PCP genes (see Table S1, adapted from (Tissir & Goffinet, 2013)) in addition to genes specifically implicated in vertebrate PCP signaling. Mutations in these "accessory" PCP components such as Wnt5 (Kilian et al., 2003; Qian et al., 2007) and Wnt11 (Heisenberg et al., 2000; Tada & Smith, 2000) the Fzd coreceptor Glypican 4 (Gpc4) (Ohkawara, 2003; Topczewski et al., 2001), Scribble (Bilder & Perrimon, 2000; Montcouquiol, Rachel, Lanford, Copeland, & Kelley, 2003a; Murdoch et al., 2003; Walsh, Grant, Morgan, & Moens, 2011), and Protein Tyrosine Kinase 7 (Ptk7) (Lu et al., 2004) results in many PCP phenotypes, some analogous to those seen in *Drosophila*, and others specifically seen in vertebrates.

In contrast to the static wing epithelium in *Drosophila,* many of the core PCP proteins in vertebrates play a role in dynamic phenotypes, involving individual and/or groups of cells conducting temporally and spatially coordinated movements. Mutations in *fzd3, fzd6,* (Mitchell et al., 2009; Y. Wang, Badea, & Nathans, 2006a; Y. Wang, Guo, & Nathans, 2006b), *dvl1-3* (Park et al., 2008), *vangl2* and *scrib* (Montcouquiol, Rachel, Lanford, Copeland, & Kelley, 2003a; Walsh et al., 2011) result in defective hair follicle orientation (*fzd3, fzd6, dvl1-3*) auditory-vestibular hair cell alignment (*fzd3, fzd6, dvl1-2, vangl2, scrib*), and loss of floorplate polarity (*vangl2, scrib*). These vertebrate PCP phenotypes occur in static epithelial tissue that are

reminiscent of invertebrate PCP phenotypes and require PCP signaling to direct the orientation and organization of cellular structures. However, in addition to these static phenotypes, vertebrate PCP mutants possess defects in dynamic developmental processes. These phenotypes include convergent-extension cell movements, neurulation, directed cranial facial motor neuron migration, and axon pathfinding.

PCP in convergent extension and neurulation

Coordinated cell movements in early vertebrate development have been shown to require PCP components. Convergent extension (CE) is one of these movements and was initially described in *Xenopus* (Keller, Danilchik, Gimlich, & Shih, 1985). During gastrulation, cells converge along the mediolateral (ML) axis (convergence), simultaneously narrowing the tissues mediolaterally, and elongating the body along the anterior-posterior (A-P) axis (extension). This intercalation can occur within a specific cell layer of a tissue (mediolateral intercalation) and also across layers in the same tissue (radial intercalation) (reviewed in (Solnica-Krezel & Sepich, 2012)). CE has been shown to occur by two mechanisms: i) polarized cell divisions and ii) collective cell movements. Polarized cells can give rise to daughter cells that are aligned with the A-P axis, thereby extending the A-P length of the embryo (Gong, Mo, & Fraser, 2004). Additionally, CE can occur by collective cell migration. In zebrafish, cells in the lateral mesoderm during gastrulation migrate along a trajectory directed toward the dorsal midline. The population then converges with the contralateral mesodermal population and then extends the A-P axis. Those cells closer to the anterior animal pole possess a slight anterior migration bias and those closer to the vegetal pole possess a slight posterior migration bias. Therefore, the

convergence of the lateral mesoderm at the dorsal midline results in an extension of the body in both the anterior and posterior directions (Sepich, Calmelet, Kiskowski, & Solnica-Krezel, 2005).

These CE cell movements continue on into neurulation. The extension of the body results in an elongated neural plate that develops a central groove. The dorsal margins of the two walls of this groove form an invaginated U-shaped structure that eventually fuse and form the neural tube. Failure of the neural tube to fully fuse results in neural tube defects (NTDs), which ultimately result from a failure of the floorplate to undergo CE. A wider floorplate inhibits the ultimate fusion of the dorsal neural tube, leaving it open. NTDs in humans affects 1 in 1000 live births (Copp, Greene, & Murdoch, 2003). NTDs in humans and mice such as cranioarchischisis and myelomeningocele occur as a result of mutations in core PCP components *VANGL1* (Kibar et al., 2007), *VANGL2* (Doudney & Stanier, 2005), *FZD3* (Y. Wang, Guo, & Nathans, 2006b), *FZD6* (De Marco et al., 2012), *PRICKLE1* (Bosoi et al., 2011), and *CELSR1*(Allache, De Marco, Merello, Capra, & Kibar, 2012; Robinson et al., 2012). Similar CE defects and NTDs occur in frogs, (Tada & Smith, 2000; Wallingford et al., 2000), and mice (Kibar et al., 2001; J. Wang, 2006; Ybot-Gonzalez et al., 2007). Zebrafish do not have neural tube defects because the neural tube forms differently. However, mutations in these aforementioned PCP genes result in CE extension defects where the embryo is shorter along the A-P axis and wider (Jessen et al., 2002). These and other studies (reviewed in Wang and Nathans, 2007; Tissir and Goffinet, 2013) involving core PCP components and other PCP accessory proteins such as Scribble (Robinson et al., 2012; Wada, 2005) have linked PCP signaling to the cell movements of CE and neurulation.

Previous to gastrulation and CE in zebrafish, mesodermal and neuroectodermal cells are initially unpolarized with random protrusions. In response to global Wnt gradient cues, these cells then polarize along the A-P axis, restricting their protrusions to the mediolateral edges as they crawl along one another during mediolateral intercalation (Yin et al., 2008). At later stages, evidence suggests that neuroepithelial cells remain planar polarized along the A-P axis. Furthermore, Prickle has been shown to localize asymmetrically to the anterior membranes of neuroepithelial cells undergoing CE (Ciruna et al., 2006; Walsh et al., 2011) and Dsh localized along posterior membranes (Yin et al., 2008) indicating a planar polarization of these cells. Indeed, in the floorplate cells, which arise from this CE population, polarity is lost in *vangl2* and *scrib* mutants (Walsh et al., 2011).

PCP and neurodevelopment

In addition to its role in gastrulation, neurulation, auditory-vestibular hair cell alignment, and spinal cord floorplate polarity, PCP plays a significant role in other neurodevelopmental processes. This study will focus on PCP's role in neuron migration and axon pathfinding, though it has been shown to also influence neural crest migration (De Calisto, Araya, Marchant, Riaz, & Mayor, 2005; Shnitsar & Borchers, 2008) and dendritic development (Shima, Kengaku, Hirano, Takeichi, & Uemura, 2004). The most apparent example of PCP in directed cell migration is in the migration of the facial branchiomotor neurons (FBMNs) in zebrafish. These neurons, which form cranial nerve VII, migrate both tangentially and caudally (in the posterior direction, toward the tail) from their birthplace in rhombomere 4 (r4) to rhombomere 6 (r6) and 7 (r7) through the ventral neuroepithelium, adjacent to the floorplate. There, they form the facial motor nucleus

from which axons exit the hindbrain in r4 and innervate muscles in the head derived from the second branchial arch (Chandrasekhar, Moens, Warren, Kimmel, & Kuwada, 1997a). With the ability to easily visualize FBMN migration using the *islet1:GFP* transgenic zebrafish, mutations in PCP genes *fzd3a* (Wada, Tanaka, Nakayama, Iwasaki, & Okamoto, 2006), *vangl2* (Bingham, Higashijima, Okamoto, & Chandrasekhar, 2002; Jessen et al., 2002), *celsr2* (Wada et al., 2006), *scribble* (Wada, 2005), and knockdown of *prk1a* (Carreira-Barbosa, 2003) result in impaired or abolished FBMN migration. Additionally, the results of numerous transplantation experiments have revealed that PCP gene function both within the FBMNs (cell-autonomously) and in the neuroepithelial environment (non-cell-autonomously) to control the trajectory of FBMNs migration. *Frizzled3a, celsr2* (Wada et al., 2006), *pk1b* (Rohrschneider, Elsen, & Prince, 2007), *vangl2,* and *scrib* (Walsh et al., 2011) function both cell-autonomously and non-cell-autonomously in FMBN migration. However, these studies have not provided any insight on the localization of PCP genes involved in FBMN migration. PCP mutant FBMNs produce random protrusions, similar to cell protrusions before becoming polarized and undergoing CE. Much like how PCP components are asymmetrically localized in polarized CE cells, it is important to determine if PCP components possess a similar cellular localization of these components during directed FBMN migration either cell-autonomously or non-cell-autonomously.

PCP components have also been implicated in axon guidance. In mammals, mutations in *fzd3* or *Celsr3* results in elimination of major axons tracts in the cortex and the misguidance of spinal cord sensory neurons. Specifically, tracts that connect the cortex and the thalamus are absent in *Fz3* and *Celsr3* mutants (Tissir, Bar, Jossin, De Backer, & Goffinet, 2005; Y. Wang, Thekdi, Smallwood, Macke, & Nathans, 2002; Y. Wang, Zhang, Mori, & Nathans, 2006c).

Though neuroproliferation and migration in the forebrain remains unaffected by these mutations, axons enter the intermediate zone, but then fail to extend, and are subsequently degraded (Y. Wang, Zhang, Mori, & Nathans, 2006c). In the spinal cord, commissural neurons make rostral turns after crossing the midline. In *ex vivo* experiments, Wnt4-Fzd3 signaling has been shown to be sufficient to make this anterior decision (Lyuksyutova et al., 2003). It has been proposed that in the presence of a Wnt4 mRNA expression gradient, the Fzd3 receptor is utilized in these commissural neurons to guide the axon to its target in the rostral direction, somehow responding to a signaling gradient. It still remains unclear how PCP, transduced by the Fzd3a receptor, directs axon pathfinding and which specific mechanisms are utilized.

It is the aim of this study to further elucidate the role of PCP in neurodevelopment. To do so, we have selected two ares of focus. The first, is to examine the role of PCP in axon guidance of commissural neurons in the zebrafish spinal cord. The second is to examine the subcellular distribution of one PCP protein, Scribble, in migrating cells and their environment to better understand how PCP provides migratory cues. We will expound upon each aim in detail, in the following chapters.

Chapter II: Planar cell polarity genes control anterior-posterior axon guidance by Commissural Primary Ascending (CoPA) neurons in spinal cord of zebrafish

II.A. Introduction

Nervous system function depends on the precise organization of synaptic connections made during development. A critical step in the assembly of these neural circuits is the proper guidance of axons to their target cells during early neural development. Extensive work in both vertebrate and invertebrates have revealed that multiple guidance cues control the trajectory of growing axons along both the dorso-ventral (D-V) and anterior-posterior (A-P) axes of the developing neural tube.

One population of early born neurons that are guided in both the D-V and A-P axes are commissural neurons in the spinal cord. Commissural axon pathfinding has been extensively studied, implicating a range of guidance cues that control axon trajectories both ipsilaterally towards the midline and contralaterally after midline crossing. The cell somas of these neurons reside in the dorsal spinal cord, where they project their axons ventrally and enter the floorplate. From there, commissural axons will exit the floorplate on the contralateral side of the spinal cord, and then turn dorsally and anteriorly.

Molecules that guide commissural axons along the dorsal-ventral axis and through the midline in zebrafish have been well identified. Commissural axons are first attracted to the ventral midline by floorplate-derived chemo-attractants (such as Netrins and Shh), and permissive cell-contact cues enable their entry to the floorplate. While navigating the floorplate, commissural axons lose their sensitivity to these attractants and acquire responsiveness to various midline repellents such as Slit, Ephrin and Semaphorin family members (reviewed in Nawabi and Castellani, 2011). The midline repellents then expel commissural axons from the floorplate.

Upon exit from the floorplate, responsiveness to anterior-posterior gradients of attractive and repulsive cues presumably guide the axons to turn rostrally (anteriorly). Recent evidence in mice suggests that the non canonical Wnt/PCP signaling pathway may influence anterior turning of axons of commissural neurons in the developing spinal cord (see below) (Lyuksyutova et al., 2003; Shafer, Onishi, Lo, Colakoglu, & Zou, 2011).

In zebrafish spinal cord, there are several interneurons that can be classified as commissural neurons in that they send axonal projections to the contralateral side through the floorplate during neural development. These include the excitatory glutamatergic neurons: commissural primary ascending (CoPAs), commissural secondary ascending (CoSAs), multipolar commissural descending (MCoDs), and unipolar commissural descending (UCoDs). Inhibitory glycinergic neurons that cross the midline include the commissural secondary ascending (CoSAs), commissural bifurcating longitudinal (CoBLs), commissural local (CoLo), commissural longitudinal ascending (CoLAs) (Bernhardt, Chitnis, Lindamer, & Kuwada, 1990; Hale, Ritter, & Fetcho, 2001; Higashijima, Mandel, & Fetcho, 2004; Kuwada, Bernhardt, &

Chitnis, 1990a; Kuwada, Bernhardt, & Nguyen, 1990b) (figure S2). All these neurons possess axons that pathfind through the floorplate into the contralateral side of the spinal cord to reach their targets.

CoPA neurons are easily distinguishable by their distinct appearance. These neurons exist on average, one per hemisegment and are the earliest commissural ascending neurons in development. CoPA neurons are born around 16-17 hpf and begin to send out their axons shortly thereafter. After emerging from the soma, the axon projects ventrally towards the floorplate of the spinal cord and enter the floorplate at approximately 18-19hpf. After crossing the midline, it exits the floorplate and enters the contralateral side of the spinal cord and then projects both dorsally and anteriorly. By 20-21 hpf, the axon returns to the dorsal level of other contralateral CoPA somas and continues anteriorly by joining the dorsal longitudinal fasciculus (DLF). The axon reaches the hindbrain by 27 hpf (Kuwada, Bernhardt, & Nguyen, 1990b). Similarly, the CoSAs following a similar pathfinding route as the CoPAs. However, CoSA cell bodies are slightly more ventral in comparison to CoPA cell bodies. Additionally, CoSAs appear later in development than CoPAs and possess smaller dendrites and axons. The axons of CoSAs do not project into the hindbrain, rather appear to extend no more than ten segments from the soma (Bernhardt et al., 1990).

Functionally, CoPAs in zebrafish have been implicated in the glutamatergic-driven touch response of the tail and receive sensory input from Rohan-Beard (RB) neurons (Gleason et al., 2003; Pietri, Manalo, Ryan, Saint-Amant, & Washbourne, 2009). CoPA neurons send projections into the contralateral rostral (anterior) spinal cord where they synapse with descending interneurons, such as the circumferential descending interneurons (CiD), that make up the

contralateral motor network (Bernhardt et al., 1990; Hale et al., 2001; Pietri et al., 2009; Saint-Amant & Drapeau, 2001). The resulting circuit is responsible for contralateral contraction in response to touch on the ipsilateral side (Pietri et al., 2009).

Due to their isolated single cell nature and their distinct visual appearance, CoPAs serve as a simple system to study axon pathfinding. As previously described, CoPAs make numerous pathfinding decisions on the way to their targets. These include a (1) ipsilateral ventral projection, (2) midline crossing, and (3) a dorso - (4) anterior projection (figure 1A) (Kuwada, Bernhardt, & Nguyen, 1990b). A recent study has found that the ventral projection and midline crossing rely on attractive netrin-dcc signaling and repulsive slit-robo signaling. Knockdown and knockout of *dcc* (deleted in colorectal cancer) results in CoPA neurons unable to make a midline cross, while still making anterior projections. Additionally, *robo2* knockout results in CoPA neurons unable to escape the ventral midline and do not pathfind dorsally after crossing. Again, anterior pathfinding is left unhindered (Bonner et al., 2012). This suggests that CoPA axon D-V patterning is guided by netrin-dcc and slit-robo signaling while the mechanisms of A-P axon pathfinding are independent of this signaling pathway.

Recent insights in mouse commissural axons have implicated non-canonical Wnt/PCP signaling as a means to establish and guide A-P pathfinding. In *ex vivo* spinal cord experiments, knockout of the Wnt4 receptor *fzd3* resulted in random pathfinding of commissural neurons after exiting the floorplate (Lyuksyutova et al., 2003). Additional *in situ* evidence has shown a Wnt4 A-P expression gradient in floorplate cells, with relatively higher levels of expression in more anterior spinal sections (Lyuksyutova et al., 2003). This evidence suggests that Wnt4 signaling is sufficient to guide axons, yet it remains unanswered if indeed the same signaling mechanism is

utilized *in vivo*. As it is known that the core PCP component Fzd3 is required in anterior axon guidance, the question remains what role PCP plays in commissural axon pathfinding and whether or not such mechanisms are cell-autonomous or non-cell-autonomous *in vivo*. This study aims to provide further evidence for the role of PCP signaling in the anterior guidance of these CoPA neurons, identify other potential PCP components in anterior axon guidance, and propose models that describe the signaling mechanisms of CoPA axon pathfinding.

II.B. Results

CoPA axon pathfinding requires PCP components Fzd3a, Vangl2, and Scrib

To visualize CoPA neurons, immunostaining was conducted using the 3A10 antibody as previously described (Bonner et al., 2012). Immunostaining was performed on fixed embryos at 33hpf when CoPA pathfinding is complete. At this developmental timepoint, there are numerous commissural neurons present in the spinal cord (figure S2), but only CoPAs were labeled with the 3A10 antibody (figure S2) (Bernhardt et al., 1990; Kuwada, Bernhardt, & Chitnis, 1990a; Kuwada, Bernhardt, & Nguyen, 1990b). Neurons labeled by 3A10 were determined to specifically be CoPAs by the following criteria: (1) neuron somas were furthest dorsal in the spinal cord, proximal to the DLF; (2) after crossing the midline, the axon projects dorso-anteriorly for 1-2 segments before joining the DLF at the level of the neuron soma on the contralateral side; (3) axons projected more than 10 segments; and (4) possess no branches (figure 1A and S2). Based on these criteria and what has been previously described, the 3A10 stained neurons were consistent with described CoPA characteristics (Bernhardt et al., 1990; Bonner et al., 2012; Kuwada, Bernhardt, & Chitnis, 1990a; Kuwada, Bernhardt, & Nguyen,

1990b). In order to conduct consistent analysis, only CoPA neurons below the 9th somite were analyzed. Wild type embryos showed no CoPA pathfinding defects.

In order to determine if commissural axon A-P guidance mechanisms described in mammalian systems are conserved in zebrafish, CoPA pathfinding was analyzed in *fzd3a*$^{rw689/689}$ homozygous mutants. The *fzd3a*$^{rw689/rw689}$ allele contains a missense point mutation in the second extracellular cysteine-rich domain (CRD), preventing Fzd3a$^{rw689/rw689}$ protein from associating with the membrane (Wada et al., 2006). In *fzd3a*$^{rw689/rw689}$ homozygous mutants, CoPA neurons undergo normal D-V guidance, with all axons properly crossing the midline through the floorplate (figure 1 D,E). However, once axons exited the floorplate, 41.8% of CoPAs in *fzd3a*$^{rw689/rw689}$ homozygous mutants failed to project anteriorly, with 3.8% stalling after pathfinding dorsally, and 38.0% (figure 1 L) projecting posteriorly (n = 79). This finding is consistent with previous *ex vivo* reports (Lyuksyutova et al., 2003) and reinforces CoPAs (Bonner et al., 2012) as a zebrafish single cell model for commissural pathfinding *in vivo*.

To determine whether other PCP components other than fzd3a play a role in A-P guidance decisions of CoPA axons, we examined CoPA neurons in other zebrafish planar polarity mutants. Embryos from homozygous mutants for *vangl2*$^{m209/m209}$, *pk1b*$^{fh122/fh122}$, and *scrib*$^{rw468/rw468}$ were analyzed for CoPA phenotypes as well. The *vangl2*$^{m209/m209}$ allele contains a 13 base pair insertion of intronic sequence that results in a frameshift mutation at Ala 441 and prematurely terminates translation. Homozygous *vangl2*$^{m209/m209}$ embryos are non-viable past 5 days, exhibit a characterized CE defect, and a FBMN migration defect (Jessen et al., 2002). The *scrib*$^{rw468/rw468}$ allele contains a point mutation that results in a pre-mature stop codon in the leucine rich repeat (LRR) domain of scrib. Homozygous *vangl2*$^{m209/m209}$ mutants and

homozygous *scrib*$^{rw468/rw468}$ mutants both display several planar polarity phenotypes including a CE defect, a defect in FBMN migration, and a defect in the planar polarity of floorplate cells in the neural tube. It should be noted that *scrib*$^{rw468/rw468}$ mutants only display a partial CE defect (Wada et al., 2005). It is proposed that *scrib* is a required PCP component both cell-autonomously and non-cell autonomously (Wada, 2005; Walsh et al., 2011). Whereas *vangl2* and *scrib* are expressed ubiquitously in the developing nervous system, *pk1b* is expressed only in distinct cell types including FBMNs, but has little expression in hindbrain and spinal cord neuroepithelial cells (Mapp et al., 2011). The *pk1b*$^{fh122/fh122}$ allele possesses a missense mutation in the farnesylation motif (CAAX domain) C869F that is predicted to abrogate its function (Mapp et al., 2011). Indeed, homozygous *pk1b*$^{fh122/fh122}$ have a FBMN migration defect, but do not exhibit a CE defect nor mispolarized floorplate cells (Mapp et al., 2011).

In homozygous *vangl2*$^{m209/m209}$, *scrib*$^{rw468/rw468}$, and *pk1b*$^{fh122/fh122}$ mutants, we observed that CoPA neurons undergo normal D-V pathfinding, with all neurons properly crossing the midline through the floorplate (figure 1D-K). After exiting the floorplate (data not shown), 34.0% of CoPA axons in *vangl2*$^{m209/m209}$ mutants (n = 94) (figure 1F,L) and 41.3% of CoPA axons in *scrib*$^{rw468/rw468}$ mutants (n = 104) (figure I,K,L) did not pathfind properly in the anterior direction. In *vangl2*$^{m209/m209}$ mutants, 4.3% of CoPA axons stalled and 29.8% projected in the posterior direction. In *scrib*$^{rw468/rw468}$ mutants, 4.8% of CoPA axons stalled and 36.5% projected in the posterior direction (figure 1L). CoPAs in *pk1b*$^{fh122/fh122}$ mutants possessed no pathfinding defect and appeared wild type (figure 1G,H,L). This is consistent with the lack of *pk1b* expression in the spinal cord (Mapp et al., 2011)

In addition to analyzing homozygous mutants, we analyzed CoPA pathfinding in embryos that were heterozygous for mutations in the PCP components *fzd3a, vangl2,* and *scrib*. Heterozygous embryos *vangl2*$^{m209/+}$ and *scrib*$^{rw468/+}$ did not possess any CoPA pathfinding defects and appeared wild type. Interestingly, CoPAs in *fzd3a*$^{rw689/+}$ mutants had a significant anterior-posterior pathfinding defect (15.3%, n = 68), albeit not as large as *fzd3a*$^{rw689/rw689}$ mutants (figure 2). Taken together, these data support the idea that PCP signaling is required for A-P guidance decisions by CoPA axons.

Anatomy and patterning of CoPAs and spinal cord remain intact in PCP mutants

To assess the possibility that the CoPA pathfinding phenotype is caused by the alteration of the spinal cord environment or by midline crossing defects, the length CoPA commissures were measured in wild type, *fzd3a*$^{rw689/rw689}$, and *scrib*$^{rw468/rw468}$ mutants (figure 3A-C). Since *vangl2*$^{m209/m209}$ embryos possessed a severe CE defect (Jessen et al., 2002) which results in a shortening of the entire body along the A-P axis, they were not measured. The mean wild type commissure length was 14.23 ± 0.80μm (n = 79) , mean *fzd3a*$^{rw689/rw689}$ commissure length was 15.78 ± 1.28μm (n = 57), and mean *scrib*$^{rw468/rw468}$ commissure length was 12.84 ± 0.80μm (n = 79). There was no significance difference in mean commissure length across phenotypes (WT-*fzd3a*$^{rw689/rw689}$ $p = 0.2554$; WT-*scrib*$^{rw468/rw468}$ $p = 0.2687$) (figure 3D) suggesting that midline and commissure anatomy is unaffected by A-P pathfinding defects.

PCP components influence CoPA axon pathfinding before midline crossing

In mice, commissural neurons do not respond to A-P cues before crossing the midline (Lyuksyutova et al., 2003). In contrast, we find that in zebrafish, CoPA axons have begun to respond to A-P cues, indicated by an appearance of an A-P bias of the pre-crossing axon in the anterior direction. We quantified the anterior-vertical-posterior directional bias of the ventrally projecting pre-crossing axons in wild-type and PCP mutants. We placed a line perpendicular to the A-P axis at each CoPA axon hillock. A second line was drawn from the axon hillock to the point of entry into the midline. If the angle between the two lines was beyond 2º to the left, the axon was marked as anteriorly biased, or 2º to the right, the axon was marked as posteriorly biased. If the axon did not exceed 2º in either direction, it was quantified as vertical, indicating a nearly perpendicular-ventrally projecting CoPA axon prior to midline crossing (figure 4A-D). In wild type, 45.8% of pre-crossing axons projected anteriorly, 31.8% possessed no A-P bias, and 22.4% projected posteriorly. Pre-crossing axons in *fzd3a*$^{rw689/rw689}$ *($p < 0.01$), vangl2*$^{m209/m209}$ (*$p < 0.01$),* and *scrib*$^{rw468/rw468}$ *($p < 0.05$)* displayed a statistically significant difference in the directional bias of these axons, with a decrease in the percentage of anteriorly projecting axons and an increase in vertically or posteriorly projecting axons (figure 4E). Once again, *pk1b*$^{fh122/fh122}$ mutants were not different than wildtype. These data support the proposition that PCP components influence CoPA axon pathfinding both before and after crossing the midline.

II.C. Discussion

Sensory neurons in the spinal cord receive signals from peripheral sensory receptors and send signals to the rostral spinal cord and the brain to process these signals that result in specific

stimulus-response behaviors. During development, these axons must pathfind far distances in order to synapse on their correct targets. The zebrafish spinal cord provides a simplified system in which axon pathfinding mechanisms can be easily visualized and studied. Study of the CoPA neurons of zebrafish provide a simple single cell system in which to study these pathfinding mechanisms.

Accumulating evidence supports the idea that planar polarity signaling plays an important role in numerous aspects of neural development. In vertebrates, Frizzled3 loss-of-function mutants possess a failure of the neural tube to close, a loss of planar polarity in the inner ear (Montcouquiol et al., 2006; Y. Wang, Guo, & Nathans, 2006b) and a loss of FBMN migration (Wada et al., 2006). Much like *fzd3*, vertebrate loss-of-function *vangl2* and *scrib* mutants also possess a failure of the neural tube to close (Doudney et al., 2005; Murdoch et al., 2003; Ybot-Gonzalez et al., 2007), a loss of planar polarity in the inner ear (Montcouquiol, Rachel, Lanford, Copeland, & Kelley, 2003a), and a loss of FBMN migration (Jessen et al., 2002; Wada, 2005; Walsh et al., 2011). Previous studies have also highlighted a role for Fzd3 in axon pathfinding. For instance, Fzd3 mutants in mouse possess a loss of numerous central nervous system tracts including the corticospinal tract, fornix, internal capsule, stria medullaris and terminalis. Additionally, there is a loss of the anterior and hippocampal commissures, as well as the corpus callosum (Y. Wang et al., 2002; Y. Wang, Zhang, Mori, & Nathans, 2006c).

Recent reports have supported the notion that planar polarity signaling controls anterior-posterior guidance of commissural neurons. In mice, commissural neurons pathfinding after midline crossing becomes randomized in the A-P axis in mouse Frizzled3 mutants (Lyuksyutova et al., 2003). Moreover, a similar defect in anterior turning of commissural axons was observed

in Vangl2 and Celsr3 mutant mice (Shafer et al., 2011; Onishi et al., 2013). In this study, we examined the role of planar polarity signaling in controlling the axon pathfinding of CoPA neurons in the spinal cord of zebrafish. We show that Fzd3a and Vangl2 are also required for A-P guidance of CoPA axons, since approximately 45% of CoPAs in these mutants fail to properly turn anterior after midline crossing. Heterozygous *fzd3a*$^{+/rw689}$ embryos also exhibit a CoPA axon pathfinding defect, though it is a less severe phenotype. This finding suggests that correct CoPA pathfinding is sensitive to the levels of Fzd3a protein. We also show for the first time that another PCP protein, Scrib, is essentially required for the anterior pathfinding of CoPA axons.

Importantly, our data also support previous results that the A-P guidance cues that control CoPA pathfinding are independent from D-V guidance cues. In zebrafish, CoPAs utilize Netrin-DCC and Slit-Robo signaling to pathfind towards and away from the midline, respectively (Bonner et al., 2012). Loss function of Netrin-DCC and Slit-Robo causes defects in midline crossing, dorsal-ventral axon growth, and commissural length defects. In *fzd3a*$^{rw689/rw689}$ mutants, however, these D-V commissural defects are not present and there are no changes in D-V axon growth towards and away from the midline. Thus, our results suggest that the signaling pathways that mediate D-V guidance and A-P guidance are independent of one another.

In mice, PCP signaling only influences the anterior turning of commissural axons after they have crossed the midline. Even in the presence of exogenous Wnt ligands applied to an *ex-vivo* mouse spinal cord preparation, commissural neurons fail to be attracted until after they have crossed the midline (Lyuksyutova et al., 2003; Shafer et al., 2011). In contrast, we have shown that zebrafish CoPA axons respond to A-P cues on both the prior to and after crossing the floorplate. While the majority of anterior turning occurs after midline crossing, we measured an

increase in the posterior bias of pre-crossing CoPA fibers in *fzd3a, vangl2,* and *scrib* mutants. This is consistent with a previous report that demonstrated that CoPA axons largely pathfind anteriorly even in midline crossing is blocked when the Netrin-receptor DCC is knocked down (Bonner et al., 2012).

Where is planar cell polarity required in order to guide CoPA axons? Formally, PCP genes could be required either in the CoPA growth cone or they could function in the environment to control CoPA axon trajectory. Evidence in mice support a role for Wnt-Frizzled signaling at the level of the growth cone. In the growth cones of mouse, commissural neurons, cultured *in vitro*, respond to the presence of Wnt5a , and in doing so, FZD3 is endocytosed at the tips of filopodia and subsequently, an increase in aPKC activation and growth cone turning occurs (Shafer et al., 2011; Onishi et al., 2013). Additionally, in the tips of filopodia, it is found that Vangl2 is enriched and may promote the internalization of the Wnt receptor, Frizzled3 at the growth cone, leading to PCP signaling (Shafer et al., 2011; Onishi et al., 2013). Although exogenous Wnt ligands have been shown to direct growth cone turning of commissural axons (Lyuksyutova et al., 2003), no loss-of-function study has yet clarified a role for Wnt ligands *in vivo*.

Additionally, these findings do not address the potential role of the cellular environment, through which these CoPA axons are pathfinding and how the *in vivo* environment may guide pathfinding through PCP mechanisms. In PCP-guided FBMN migration, it has been shown that the PCP components Fzd3a, Vangl2, and Scrib can act both cell-autonomously and non-cell-autonomously (Wada et al., 2006; Walsh et al., 2011). Cell-autonomous cues act within a neuron, creating and maintaining an internal polarity while non-cell-autonomous cues act to polarize the

environment, which neurons may "read" and align to in order to guide their directed migration. In Vangl2 and Scrib mutants, the neuroepithelium loses its anterior-posterior polarity (Walsh et al., 2011) and as such, it suggests that CoPAs are pathfinding through an unpolarized neuroepithelium in *vangl2* and *scrib* mutants. Although there is evidence that Fzd3a acts non-autonomously in the environment to control FBMN migration (Wada et al., 2006) there has yet to be a study that demonstrates direct evidence that a *fzd3a* mutant neuroepithelium environment is unpolarized. From our analysis of these PCP components, we are able to propose two models of A-P guidance, describing growth cones responding to cell-autonomous or non-cell-autonomous signaling mechanisms (figure 5).

Cell-autonomous and non-cell-autonomous models of CoPA axon pathfinding

With these data, we describe two potential models to explain PCP mechanisms guiding CoPA axon pathfinding in the spinal cord. A cell-autonomous model illustrates PCP mechanisms acting within the CoPA (figure 5A) growth cone. A proposed Wnt-gradient, strongest on the anterior end, is present through the spinal cord. mRNA expression data in mice has shown that Wnt4 is expressed in a gradient with high levels on the anterior end of the embryo, with a gradual caudal decrease in the floorplate of the spinal cord (Lyuksyutova et al., 2003). The Wnt-receptor Frizzled3a is present in the membranes of the growth cones and responds to this Wnt-gradient. Vangl2-mediated internalization of Frizzled3a in the presence of this Wnt gradient directs these filopodia toward the source of the Wnt gradient and thereby guides the axon to its target (Onishi et al., 2013; Shafer et al., 2011). In a *fzd3a* mutant, the CoPAs do not possess functional Fzd3a, and cannot respond to the presence of a Wnt gradient, resulting in a

randomization of A-P guidance. In other PCP mutants, loss-of-function Vangl2 or Scrib results in a loss of Frizzled3a regulation internalization, and thereby cannot respond to the presence of a Wnt gradient, thus also resulting in a similar randomization of A-P guidance.

A non-cell-autonomous model illustrates PCP mechanisms acting in the neuroepithelial environment through which the CoPA neuron pathfinds (figure 5B & C), similar to proposed models in FBMN migration (Wada, 2005; Wada et al., 2006; Walsh et al., 2011). A global cue acts upon the neuroepithelial cells (perhaps through Wnt signaling) and polarizes the neuroepithelium in the A-P direction by the asymmetrical localization of Fzd3a and Vangl2 complexes within the neuroepithelium. This polarity is communicated inter-cellularly through classic cell-cell PCP signaling mechanisms in epithelial tissue. The CoPA neuron thereby "reads" this polarized epithelium and aligns itself on the ipsilateral and contralateral sides of the spinal cord and pathfinds correctly to its target. In PCP mutants, the neuroepithelium is unable to align and polarize itself in relation to global cue and the CoPA axons are pathfinding through an unpolarized environment resulting in a randomization of A-P guidance. These descriptive models are not necessarily mutually exclusive, and may potentially occur concurrently to guide axon pathfinding.

II.D. "Pathfinding" forward: Future Directions

Identification of other PCP components *in vivo* in CoPA axon guidance

Here we have shown that Fzd3a, Vangl2, and Scrib are PCP components that are required in zebrafish CoPA axon pathfinding. We have also shown that Pk1b is not required for CoPA axon pathfinding. To fully understand PCP's role in CoPA axon guidance, it is necessary to

determine what other known PCP components may also be required for *in vivo.* Such components include, but are not limited to, Pk1a (Thisse et al., 2005), Pk2 (Thisse et al., 2004), Celsr (Shafer et al., 2011; Tissir et al., 2005; Wada et al., 2006), Dvl (Onishi et al., 2013; Shafer et al., 2011), Gpc4 (Topczewski et al., 2001), Ptk7 (Hayes, Naito, Daulat, Angers, & Ciruna, 2013; Lu et al., 2004), and Wnts (Lyuksyutova et al., 2003) which have all been previously shown to be required for many PCP dependent neurodevelopmental processes in vertebrates. Though again, much evidence has been presented describing Wnt gradients to be sufficient in guiding commissural pathfinding (Lyuksyutova et al., 2003; Onishi et al., 2013; Shafer et al., 2011; Zou, 2012), there has been little evidence suggesting that such Wnt protein gradients exist *in vivo* or that these mechanisms are in fact utilized by these neurons. A combination of functional knockdown or genetic knockout analysis, similar to what was previously described in this work, should be sufficient to determine what other components maybe required for CoPA A-P axon guidance.

Utilization of tbx16:GFP transgenic zebrafish to molecularly image CoPA axon guidance

There is currently no understanding of the molecular dynamics of CoPA growth cone guidance and these PCP components *in vivo.* A line of tbx16:GFP transgenic zebrafish created by Wells et al., has resulted in the serendipitous expression of GFP within the CoPA neurons (Wells, Nornes, & Lardelli, 2011). This fortunate result opens the potential for live imaging of these neurons as they pathfind in living embryos. In conjunction with other molecular and genetic tools and manipulations, we will aim to image the molecular dynamics of these PCP components *in vivo* by using mCherry-tagged to PCP proteins of interest and the high speed acquisition rates

of spinning disk laser confocal microscopy. Such experiments could potentially yield valuable information on whether the dynamic cycling of components Vangl2 and Fzd3 seen *in vitro* (Onishi et al., 2013; Shafer et al., 2011) are seen *in vivo*, as there has been little or no evidence of such molecular dynamics occurring in live embryos.

Determination of cell-autonomous/non-cell-autonomous mechanisms in CoPA axon guidance

It is unclear whether PCP components are acting cell-autonomously or non-cell-autonomously in these CoPA axons as they pathfinding during development. In order to provide insight on the nature of these mechanisms, we propose the utilization of transplant experiments. Transplant experiments have been previously utilized to determine the cell-autonomous/non-cell-autonomous nature of PCP components in the migration of FBMNs (Carmany-Rampey & Moens, 2006; Kemp, Carmany-Rampey, & Moens, 2009; Walsh et al., 2011). In combination with the tbx16:GFP transgenic zebrafish, which express GFP in CoPA neurons (Wells et al., 2011), creation of chimeric embryos containing wild type CoPAs (expressing in the background tbx16:GFP) in PCP mutant hosts (*fzd3a*$^{rw689/rw689}$, *vangl2*$^{m209/m209}$**,** and *scrib*$^{rw468/rw468}$) will provide evidence of cell-autonomous/non-cell-autonomous requirements of these PCP genes and assess the validity of our two models.

Examination of other commissural axon guidance mechanisms (CoLA, CoLo, CoBL, etc.)

It is unknown whether or not PCP components are required for the guidance of other commissural neurons in the spinal cord of zebrafish. As described previously, there exist many

other types of neurons, both glutamatergic and glycinergic, which also send commissural axons into the contralateral side of the spinal cord (figure S2) (Bernhardt et al., 1990; Higashijima et al., 2004; Kuwada, Bernhardt, & Chitnis, 1990a; Kuwada, Bernhardt, & Nguyen, 1990b; McLean & Fetcho, 2008). The analysis of these other commissurals is important for determining what potential factors distinguish these commissurals from others that are similar. For example, Commissural bifurcating longitudinal (CoBLs) and Commissural local (CoLos) have very similar axon trajectories, with the only distinguishing factor being synaptic location (with CoLos remaining within a hemisegment). Additionally, the guidance cues of the bifurcating axons is unknown. Presumably, A-P guidance accomplished by these previously described PCP components. As they belong to the same neuron, it is curious what guides one axon branch anterior, and the other posteriorly. Initial studies on these guidance cues can be done by HRP backlabelling (described in (Bernhardt et al., 1990)) in PCP loss-of-function knockout embryos. These experiments could provide preliminary evidence on the nature of the guidance cues of these other commissurals in the zebrafish spinal cord.

Chapter III: Subcellular Localization of PCP Component Scribble in Neurodevelopment

III.A. Introduction

Scribble (Scrib) is a member of the LAP family of proteins (LRR and PDZ domain (post-synaptic density, Dlg1, and zonula-occulens 1)). It contains LRR (leucine rich repeats) domains, which function as a protein-protein interaction motif, and four PDZ domains, a multi-protein interaction domain (Bilder & Perrimon, 2000; Bilder, Schober, & Perrimon, 2002; Kallay, McNickle, Brennwald, Hubbard, & Braiterman, 2006). Scribble is thought to belong to a complex of proteins known as the Scribble Complex that includes Scrib, Disc large (Dlg), and Lethal giant larvae (Lgl) that appear to function in establishing apical-basal polarity. Mutations in *Drosophila scrib, dlg,* or *lgl* has been shown to result in loss of apical-basal (A-B) polarity, impaired cell cycle exit, and tissue, or tumor overgrowth (Bilder, 2004). For that, they have been named the neoplastic tumor suppressor genes (nTSGs). Scrib, Dlg, and Lgl have been to shown to genetically interact and loss-of-function mutants of these genes result in similar developmental phenotypes. In *Drosophila* epithelial cells, Scrib is localized, along with Dlg and Lgl to the basolateral membrane. Scrib has also been shown to play a role in invertebrate neuronal synaptic scaffolding complex by associating physically with GUK (guanylate kinases) holder and Dlg (Mathew et al., 2002).

In higher vertebrates, there is only one homologue of Scrib (Bilder, 2004; Humbert, Russell, & Richardson, 2003; Santoni, Pontarotti, Birnbaum, & Borg, 2002). In polarized epithelia in mammals, Scrib has been shown to physically associate with LgL2. Whereas loss-of-function of Scrib in fly results in phenotypes characteristically similar to a loss of apico-basal polarity (Bilder, 2000; Bilder & Perrimon, 2000; Humbert et al., 2003). Loss-of-function *scrib* mutations in vertebrates leads to many phenotypes similar to planar cell polarity (PCP) loss-of-function mutations. These include defects in convergent-extension (CE) cell movements (Wada, 2005), neurulation and neural tube defects (Murdoch et al., 2003; Žigman, Le A Trinh, Fraser, & Moens, 2011), loss of inner ear hair cell polarity and alignment (Montcouquiol, Rachel, Lanford, Copeland, & Kelley, 2003a), loss of neuroepithelial planar polarity (Walsh et al., 2011), a loss of directed neuron migration of the FBMNs (Wada, 2005), and more recently, in commissural axon pathfinding as I have shown, discussed in the previous chapter of this work. Interestingly, Scrib has been shown to physically interact with Vangl2, a core PCP component. More specifically, Vangl2 binds selectively to the second and third, or all four PDZ-domains of Scrib simultaneously (Kallay et al., 2006). Moreover, Scrib can genetically interact with Vangl2 in neural tube closure and CE movements during gastrulation. This interaction with Vangl2 potentially implicates Scrib as a component of vertebrate non-canonical Wnt/PCP signaling.

In other cell contexts, Scrib has a known function in regulating cell migration. Functionally, Scrib in cell culture has been shown to promote cell protrusions via subcellular modulation of Rac and Cdc42, which form a complex with the exchange factors βPIX and GIT1 (Audebert et al., 2004; Dow et al., 2007; Nola et al., 2008; Osmani, Vitale, Borg, & Etienne-Manneville, 2006). Other implicated Scrib interactions include the JNK signaling pathway

(Brumby & Richardson, 2003; Uhlirova, Jasper, & Bohmann, 2005) and the βPIX-GIT1 complex, which regulates Rac1, Rho GTPase, and Cdc42 signaling (Audebert et al., 2004; Dow et al., 2007; Nola et al., 2008; Osmani et al., 2006). Given the fact that the βPIX-GIT1 complex can modulate focal adhesions, this implicates and links Scrib to focal adhesions and regulation of cytoskeletal dynamics (Hoefen, 2006).

The molecular function of PCP signaling is dependent on their asymmetric localization in epithelial cells. For example, the core PCP components, such as Fzd-Dsh and Vang-Pk, in *Drosophila* wing epithelium possess an asymmetrical subcellular distribution. Fzd-Dsh complexes localizes to the distal membrane while Vang-Pk complexes localizes to the proximal membrane. This subcellular asymmetry is translated into polarity in these epithelial cells (figure S2) (Das et al., 2004; Giese et al., 2012; Y. Guo et al., 2013; Shimada et al., 2006; H. Strutt & Strutt, 2005; 2008; Tree et al., 2002). Importantly, genetic loss of any one core PCP component leads to a loss of asymmetric distribution in the other proteins, indicating that their subcellular localization is mutually dependent on one another.

Scrib's subcellular localization has provided evidence for the roles it plays in different cell contexts. In invertebrate epithelium, Scrib localizes specifically to the apical-basal (AB) border at the septate junction and acts as a determinant for the localization of zonula adherens to the apical membrane (Bilder, 2000; Bilder et al., 2002; Bilder & Perrimon, 2000). In migration assays, Scrib localizes to the leading edge in migrating cells and forms a complex with βPIX and GIT1 to increase cell protrusions by Rac, Rho, and Cdc42 signaling (Audebert et al., 2004; Dow et al., 2007; Nola et al., 2008; Osmani et al., 2006).

In vertebrate epithelial cells, Scrib is also localized to the basolateral surface and adherents junctions (Yoshihara et al., 2011). However, its localization in migrating cells is less well characterized. As Scrib is required for many PCP-dependent processes, it is of interest to characterize its subcellular localization. To do so, an antibody was generated in mouse against the C-terminus of zebrafish Scrib protein. The aim of this study is to determine that our monoclonal IgG2b antibody is specific against Scrib. Subsequently, the antibody will be used to localize the subcellular distribution of Scrib in specific tissues and cells in zebrafish that are affected in planar cell polarity mutants. These include the the neuroepithelial cells, cells undergoing CE movements, and migrating FBMNs. Lastly, our goal is to determine if the subcellular localization of Scrib is dependent on other core PCP components.

III.B. Results

Monoclonal antibody is specific for Scrib

To generate an antibody, the Walsh lab fused the C-terminus of zebrafish Scrib with GST and expressed in bacteria. Glutathione-sepharose beads were used to purify the protein and GST-(CT)SCRIB protein was injected into mouse. The whole blood serum was then extracted and initially tested for antibodies specific to Scrib by western blot and immunostaining (data not shown). Individual clones were harvested from the spleen and individual supernatants from clones were tested for specificity by western blot. An IgG2b clone was selected for its specificity in western blot (figure 6A). In lane 2 of figure 6A, a band corresponding to endogenous Scrib at around 250kD can be seen in lysates from wild type embryos. This band is absent in lysates from maternal-zygotic (MZ)-*scrib*$^{rw468/rw468}$ embryos. We then tested this antibody in immunostaining

conditions. In wild types, Scrib immunostaining is present in an isl1:GFP background co-stained for GFP (figure 6C). In contrast, Scrib immunostaining is absent in MZ-*scrib*$^{rw468/rw468}$ embryos (figure 6F) while staining for GFP present in unmigrated FBMNs (figure 6B,D,E,G). These results indicate that our monoclonal antibody generated against the C-terminus of zebrafish Scrib is specific in both western blotting and immunocytochemistry.

Scrib in vertebrate epithelium localizes to Apico-Basal boundary

Scrib in epithelial cells of invertebrates has been shown to localize to the apical-basal boundary at the septate junction as a determinant for the location of zonula adherens to the apical membrane (Bilder, 2000; Bilder et al., 2002; Bilder & Perrimon, 2000). To determine the localization of Scrib in zebrafish epithelial cells, Tg(β-actin:EGFP-CAAX) embryos were fixed at 24hpf and immunocytochemistry staining against GFP and Scrib was conducted. Scrib staining in epithelium of the otic vesicles was localized to the basolateral membranes (figure 7D). Scrib was absent from the apical membrane of these otic epithelial cells (figure 7D,E). Additionally, distinct Scrib puncta were detected at the AB border of these epithelial cells (figure 7D). To determine if the otic vesicle was planar polarized, immunocytochemistry was conducted against F-actin (phalloidin) and acetylated tubulin to determine the location of cilia in the otic vesicles (figure 7A,B). The cilia are located in the center of otic epithelial cells and do not appear to be planar polarized in a specific direction (figure 7A). There is no change in the location of cilia in MZ-*scrib*$^{rw468/rw468}$ (figure 7B). This immunocytochemistry study indicates that Scrib, in non-planar-polarized vertebrate epithelial tissue, is localized to the basolateral membrane, not the apical membrane, and as distinct puncta at the AB border.

Scrib localizes to cell membranes in mesoderm undergoing convergent-extension movements

The *scrib*$^{rw468/rw468}$ allele contains a point mutation that results in a stop codon in the LRR domain of *scrib*. Homozygous MZ-*scrib*$^{rw468/rw468}$ mutants possess a partial CE defect (Wada, 2005). As Scrib plays an important role in CE movements, we immunostained for Scrib in tissues undergoing CE movements. At the tail bud stage, the dorsal mesoderm is undergoing CE (figure 8A, F-H). These cells of the dorsal mesoderm possess a more elongated appearance (figure 8F) in comparison to cells not undergoing CE movements (figure 8C). In cells in the lateral mesoderm, Scrib localizes to the membrane (figure 8D,E) as well as in cells in the dorsal mesoderm (figure 8G,H). Scribble also localizes as foci on the membrane in both of these tissues. We did not detect any inherent asymmetry in Scrib localization within cells of the lateral mesoderm, nor in cells undergoing CE at the dorsal mesoderm.

Scrib in migrating FBMNs localizes to membranes as distinct puncta

Scrib is required for the caudal-tangential migration of the facial branchiomotor neurons (FBMNs) from r4 to r6 and r7 (Wada, 2005) and in migrating cells *in vitro*, has been shown to localize to the leading edge (Audebert et al., 2004; Dow et al., 2007; Nola et al., 2008; Osmani et al., 2006). As the localization of Scrib is unknown in migrating cells, immunocytochemistry was conducted on Tg(isl1:GFP) embryos , that express GFP in cranial motoneurons, including FBMNs, at 18hpf when FBMNs begin to migrate and at 24hpf, during which FBMNs are undergoing migration through r5 (figure 9). Scrib staining again localizes to the membrane of

migrating FBMNs and as distinct puncta along the membranes. In should be noted that Scrib also appears to localize in the same manner in the surrounding neuroepithelium (figure 9E). The localization does not appear to be inherently asymmetric, nor does there appear to be any distinct localization to protrusions or lamellipodia (figure 9B,E).

Scrib puncta distribution along sites of neuron-neuron contacts is influenced by Prk1b

In PCP signaling, genetic loss of one component results in alteration of the distribution of the other PCP components (D. Strutt, 2003). As Scrib is required for the caudal-tangential migration of FBMNs, during which, neurons are in close contact with one another (Chandrasekhar, Moens, Warren, Kimmel, & Kuwada, 1997b), it was of interest to characterize the specific subcellular localization of Scrib at these points of contact and determine if the localization changes in other PCP mutants possessing a similar phenotype. The number of puncta specifically along a length of membrane FBMN-FBMN contacts at 24hpf (figure 10A-D) was quantified in wild type, *pk1b*$^{fh122/fh122}$ and *vangl2*$^{m209/m209}$ mutants. The mean number of puncta for each membrane length along FBMN-FBMN contact in wild type was 5.96 puncta (n = 51), in *pk1b*$^{fh122/fh122}$ was 3.42 (n = 38), and in *vangl2*$^{m209/m209}$ was 6.63 (n = 38) (figure 10E). A student's t-test was conducted to compare mean puncta. There was a significant difference in means between wild type and *pk1b*$^{fh122/fh122}$ ($p < 0.0001$). There was no significant difference between wild type and *vangl2*$^{m209/m209}$. Due to the varying lengths of these points of FBMN-FBMN contact, the puncta count was normalized by the length of the measured membrane. Normalizing produced a distribution that could be considered a normal distribution. The normalized mean number of puncta per unit (μm) membrane length along FBMN-FBMN contact in wild type was

0.644 puncta/µm (n = 51), in *pk1b*$^{fh122/fh122}$ was 0.464 puncta/µm (n = 38), and in *vangl2*$^{m209/m209}$ was 0.706 puncta/µm (n=38) (figure 10F). A student's t-test was conducted to compare mean puncta/µm. There was a significant difference in normalized means between wild type and *pk1b*$^{fh122/fh122}$ ($p < 0.0001$). There was no significant difference between wild type and *vangl2*$^{m209/m209}$. These data indicate that the subcellular localization of Scrib in FBMNs along points of contact with other FBMNs is significantly altered by loss-of-function of Pk1b.

III.C. Discussion

PCP components play an important role in neurodevelopment. The subcellular localization of many of these PCP components signals internal polarity within cells and in the environment external to the cell (D. Strutt, 2003). It is the subcellular distribution of these PCP components that is translated into polarity and through effectors, directs many development processes (Das et al., 2004; Giese et al., 2012; Y. Guo et al., 2013; Shimada et al., 2006; H. Strutt & Strutt, 2005; 2008; Tree et al., 2002). Scrib is specifically required for many aspects of neural development that require PCP signaling, including convergent-extension (CE) cell movements (Wada, 2005), neurulation (Murdoch et al., 2003; Žigman et al., 2011), inner ear hair cell polarity and alignment (Montcouquiol, Rachel, Lanford, Copeland, & Kelley, 2003a), neuroepithelial polarity (Walsh et al., 2011), and directed cell migration of the FBMNs (Wada, 2005; Walsh et al., 2011). Within cells and tissues undergoing these PCP-dependent development processes, it is of interest to understand the endogenous subcellular localization of Scrib and if such localization will help elucidate its function, as either a PCP signal or an effector. To characterize endogenous Scrib localization, we have generated an antibody against zebrafish Scrib for use in

immunocytochemistry in fixed tissues. Our findings indicate that the expression of Scrib is ubiquitous and is generally membrane-localized; however, localization varies across tissues and is influenced by some PCP components.

Our monoclonal antibody against Scrib is specific

Our control experiments have indicated that our antibody is specific against Scrib for applications in western blotting and immunocytochemistry. The absence of a signal in western blotting with our antibody in MZ-*scrib*$^{rw468/rw468}$ indicates that our antibody has minimal nonspecific binding, even though 90-100µg of protein was loaded on the gel as evidenced by the ponceau-staining of the membrane (figure 6A). Moreover, immunostaining with anti-Scrib is completely absent from MZ-*scrib*$^{rw468/rw468}$ embryos, further confirming the specificity of the antibody.

Localization of Scrib in epithelial tissues

Immunocytochemistry of the otic vesicle in zebrafish, which possesses classical epithelial characteristics, has shown that Scrib localizes to the basolateral membrane and also as distinct puncta at the apical-basal boundary. This localization is similar to that of invertebrates, where Scrib also is localized distinctly at the septate junction, just basal of the apical membrane (Bilder, 2000; Bilder et al., 2002; Bilder & Perrimon, 2000). However, Scrib is not localized to basal membranes in invertebrate epithelial tissue, while our data indicates that Scrib localizes basolaterally in vertebrates. This localization suggests that Scrib plays a role in establishing

apical-basal polarity in both invertebrates and vertebrates in epithelial tissue. In vertebrates, this may result in the proper placement of adherens junctions in epithelial tissue.

Scrib localizes to the membrane in migrating cells during development

Our results in tissues undergoing CE and not undergoing CE, and FBMNs indicates that Scrib ubiquitously localizes to the membrane, with occurrences of distinct puncta. It has been reported that Prickle is anteriorly localized in mesodermal cells undergoing convergent extension, whereas Disheveled is posteriorly localized (Yin et al., 2006). Importantly, although we observed Scrib to be localized to the membrane, we did not notice any clear asymmetric distribution between anterior or posterior membranes.

In other cell migration assays, Scrib has been shown to localize to the leading edge where it promotes actin polymerization and lamellipodial formation. In migrating FBMNs, we do not observe Scrib only at leading edges. In fact, we find that Scrib is localized along all membranes, often in bright puncta. These are not exclusive to the leading edge, but rather are found on all sides of the FBMNs, even at sites of neuron to neuron contact. The appearance of Scrib in membrane puncta could be due to several reasons. First, the staining pattern could be an artifact of fixation with trichloroacetic acid (TCA), which is known to cause protein aggregation and precipitation. Further studies testing other fixation protocols are necessary to resolve this issue. Second, the foci of Scrib staining could reflect Scribble association with other proteins complexes at or near the plasma membrane. Indeed, Scrib has four PDZ domains that may serve to scaffold other signaling proteins to the membrane (Kallay et al., 2006). Scrib has been shown to physically bind with Vangl2 at PDZ2 and PDZ3 domains (Kallay et al., 2006), and as such

may serve as an effector of PCP by interacting with Vangl2 at the plasma membrane. Lastly, there is evidence that Scribble interacting partners GIT1 and βPIX are known to associate with and modulate focal adhesion dynamics, which are necessary for proper cell migration. This suggests the intriguing possibility that Scrib puncta represent the association of Scrib with focal adhesion complexes in migration neurons.

Scrib in PCP component knockout indicates its localization is tied with PCP signaling

Our data indicates that in *pk1b* loss-of-function mutants, the localization of Scrib is significantly altered in migrating FBMNs in contact with other FBMNs. A decrease in puncta in *pk1b*$^{fh122/fh122}$ suggests that pk1b may play a role in localizing Scrib to the membrane at points of neuron-neuron contact. Interestingly, loss of another PCP protein, Vangl2, had no effect on the distribution of Scrib puncta. One interpretation of this finding is that Scrib should not be considered a core PCP protein. In fly, loss of any core component leads to a loss in the asymmetric distribution of all other core PCP proteins. Scribble could be an effector of PCP, whose localization depends not on each core member, but the activity of some of the PCP signaling proteins. Our data indicates that Scrib localization may be specifically dependent on Prickle function. Another possibility is that Scrib localization is dependent on the non-PCP functions of Prickle. Indeed, it has recently been reported that pk1b functions in FBMN migration through its nuclear localization, which acts independently of PCP signaling (Mapp et al., 2011). In this paper, they report that pk1b acts at the nucleus to regulate the neuronal transcriptional silencer REST. Therefore, in this scenario, pk1b would regulate Scrib localization secondary to its effects on REST localization and thus FBMN migration (Mapp et al., 2011).

III.D. Future Directions

Other immunological applications of our antibody

We have shown that our antibody against zebrafish Scrib is specific and as such, other immunological applications of our antibody should be utilized. Our data show that the localization of Scrib is influenced by other PCP components. Therefore, it is of interest to determine if protein levels of Scrib are affected by PCP knockout through future western blotting studies of Scrib in other PCP component mutants. Other similar studies could utilize immunoprecipitation to identify other potential binding partners of Scrib, further describing Scrib's role in development. It could also be determined if these protein interactions are affected or involve other PCP components. Such PCP components of interest include, but are not limited to Pk1b (Mapp et al., 2011), Vangl2 (Jessen et al., 2002), Fzd3a (Wada et al., 2006), Celsr (Shafer et al., 2011; Tissir et al., 2005; Wada et al., 2006), Dvl (Onishi et al., 2013; Shafer et al., 2011), Gpc4 (Topczewski et al., 2001), Ptk7 (Hayes et al., 2013; Lu et al., 2004).

Determination of a method to analyze Scrib localization in FBMN-NE contacts

Our study of Scrib localization in FBMN focused on Scrib puncta along membranes of FBMN-FBMN contacts. As such, it is of interest to determine a method to analyze Scrib puncta at sites of FBMN-neuroepithelium (NE) contact. This is of particular interest as FBMNs are migrating through the NE. Such analysis would require high resolution images and powerful image processing software due to the inconsistent shape and dynamic nature of FBMNs. A detailed analysis would require a method to measure surface area to normalize puncta counts, similar to our method of analysis. Two-photon confocal microscopy allows for deep tissue

analysis without scattered fluorescent excitation (Denk, Strickler, & Webb, 1990). Such tools may prove sufficient to acquire high-resolution images of Scrib in FBMNs to analyze localization at such a detailed level.

Scrib localization in FBMN in other PCP component knockout

Here we have shown that Pk1b influences the distribution and subcellular localization of Scrib in migrating FBMNs. It is of interest to determine if there are other PCP components that significantly influence such localization. Other PCP components such as Fzd3a and Celsr (Wada et al., 2006) are required in FBMN migration. Scrib also physically interacts with Nhsl1b (Walsh et al., 2011); mutants of Nhsl1b also have a FBMN migration defect. Analysis of the localization of Scrib in these knockouts may yield significant insight on Scrib's role in the PCP-dependent migration of FBMN, and where Scrib plays its role in this pathway. Methods to analyze such data can be similar to methods utilized in this study for FBMN-FBMN contacts.

Scrib colocalization studies

Scrib has been previously shown to interact with many other proteins such as Dlg, Lgl, and Vang (Bilder et al., 2002; Bilder & Perrimon, 2000; Kallay et al., 2006, reviewed in (Humbert, Dow, & Russell, 2006) and has been implicated as a scaffolding protein through its PDZ domains. Other proteins of interest may further describe Scrib's role in CE and FBMN migration. Candidate proteins include Nhsl1b (Walsh et al., 2011) and proteins in focal adhesion complexes. Focal adhesions (FAs) have been described in cell culture as large heterogenous protein complexes at which the intracellular matrix is linked to the extracellular matrix. Proteins

that influence FA turnover include Paxillin, which interacts with GIT-1, indirectly linking Scrib with FAs (Audebert et al., 2004; Hoefen, 2006; Matsumoto, Fumoto, Okamoto, Kaibuchi, & Kikuchi, 2010; Wehrle-Haller & Imhof, 2002). Colocalization studies with Scrib and these potential proteins in cells undergoing CE and FBMNs could directly link PCP signaling with FA turnover and control via Scrib. Such studies would also provide evidence of PCP influencing FAs *in vivo* cell migrations.

Materials & Methods

Fish Strains

All Zebrafish were maintained according to standard procedures and were staged as previously described (C. B. Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995). Wild type embryos were attained from natural mating of either Yoshi AB or ZIRC AB pairings. The fzd3a/ off-limits mutant was originally described as oltrw689 (Wada et al., 2006). The vangl2/trilobite mutant was originally described as trim209 (Jessen et al., 2002). The *pk1b*$^{fh122/122}$ was originally described by (Mapp et al., 2011). The scrib/landlocked mutant was originally described as llk^{rw468} (Wada, 2005).Scrib/llk^{rw468} and fzd3a/olt^{rw689} embryos were attained through incrossing corresponding homozygous adults. Vangl2/tri^{m209} embryos were attained through incrossing heterozygous adults. Heterozygous genotypes were attained by outcrossing mutant parents with either Yoshi AB or ZIRC AB parents. Genetic interaction embryos were attained by crossing scrib/llk^{rw468} and fzd3a/olt^{rw689} homozygous parents, scrib/llk^{rw468} homozygous and vangl2/ trim209 heterozygous parents, or fzd3a/olt^{rw689} homozygous and vangl2/tri^{m209} heterozygous parents.

Embryonic Genotyping

Vangl2/trim209 embryos were often attained from crosses that would result in embryo clutches possessing more than one genotype. As such, these embryos were genotyped before conducting any further immunocytochemistry. Vangl2/trim209 homozygous embryos were genotyped by their convergent extension phenotype. Vangl2/trim209 heterozygous and genetic interaction embryos were fixed with 4% para-formaldehyde (in 1x PBS) at 33hpf overnight at 4°C. Embryos were then washed in PBST (1 x PBS with 0.25% Triton X-100). After immunostaining, before glycerol dehydration, embryo heads were micro-dissected utilizing a Carl Zeiss stereo microscope Discovery.V8. DNA was prepped with 1x Base Solution (0.025M NaOH, 0.2mM EDTA) and incubated for 30mins at 95°C and neutralized with 1x Neutralization solution (0.04M Tris-HCl). PCR was conducted, utilizing TaKaRa ExTAQ polymerase and custom primers from Eurofins (forward: 5'-TAGGCCTGCATCTAACCAAAC; reverse: 5'-CCAAAAATGCCTGACCACAGATTC). A restriction enzyme digest utilizing AlwNI was run at 37°C overnight and then run on a 2% agarose gel stained with ethidium bromide. Heterozygous embryos possessed two bands at 70bp and 23bp.

Immunocytochemistry & Immunofluorescence

The 3A10 antibody (mouse IgG1) from DSHB was used at a concentration of 1:10 on embryos at 33hpf that had been fixed in 4% paraformaldehyde (in 1 x PBS) overnight at 4°C. Embryos were washed in PBST (1 x PBS with 0.25% Triton X-100) and permeablized with acetone and then washed again with PBST. Embryos were then blocked with PBST + 10% Goat

Serum + 10% BSA at Room Temperature for at least 1 hr. Alexa Fluor® 568 Goat Anti-Mouse IgG (H+L) Secondary Antibody (catalog number A11031, Life Technologies™) was added at a concentration of 1:200 overnight at 4°C. Embryos were washed 5 x 30 min in PBST (1x PBS with 0.25% Triton X-100) in between antibody incubations. The embryos were then dehydrated in 25%, 50%, and 75% glycerol sequentially.

The Scrib antibody (mouse IgG2b) was used at a concentration of 1:1 on embryos at various stages of development, and fixed with 2% tricholoracetic acid (TCA) (in 1 x PBS) for 1 hour at room temperature. Embryos were washed in PBST (1 x PBS with 0.25% Triton X-100) and permeablized with Acetone and then washed again with PBST. Embryos were then blocked with PBST + 10% Goat Serum + 10% BSA at Room Temperature for at least 1 hr. Alexa Fluor® 568 Goat Anti-Mouse IgG (H+L) Secondary Antibody (catalog number A11031, Life Technologies™) was added at a concentration of 1:200 overnight at 4°C. Embryos were washed 5 x 30 min in PBST (1x PBS with 0.25% Triton X-100) in between antibody incubations. The embryos were then dehydrated in 25%, 50%, and 75% glycerol sequentially.

Microscopy

For CoPA and SCRIB FBMN analysis, after immunocytochemistry and immunofluorescence, the yolks of the embryos were removed by micro-dissection and the body anterior to the yolk extension was removed. Embryo tails for CoPA analysis (the remaining posterior section) were mounted on coverslips on their sides for lateral visualization of the spinal

cords. Embryos were mounted in 75% glycerol. Wild Type and mutant embryos were visualized and mounted utilizing a Carl Zeiss stereo microscope Discovery.V8.

Confocal Microscopy

Confocal images of labeled CoPA neurons were obtained on a Carl Zeiss Spinning Disk Laser Confocal Observer.Z1 (Virginia Commonwealth University, Biology Dept.). To obtain images of the left and right sides of the spinal cord for both wild type and aberrant contralateral axon pathfinding, confocal projections were made by imaging from the location of CoPA cell bodies on one side of the spinal cord through to the CoPA cell bodies on the other side of the spinal cord. Maximum intensity projections were created in Zen Blue in order to quantify the contralateral anterior-posterior pathfinding direction of CoPA neurons.

Quantification of Caudal Axon Phenotype

CoPA neurons of embryos were screened for the rostral, medial, or caudal direction of the post-midline crossing axons. All identifiable neurons after the 8th somite were marked as to which direction along the A-P axis the axons were pathfinding. Analysis of axons before midline crossing was achieved by drawing a line perpendicular to the A-P axis at the axon hillock of each CoPA. A second line was drawn from the axon hillock to the point of entry at the floorplate. If the angle of the two lines was greater than 2º, the axon fiber was considered to have either an anterior or posterior bias. Pearson's Chi-Square Test was utilized to test percentage for statistical significance. Student's t-test was utilized to test mean proportion for statistical significance. All

statistical tests were conducted with JMP11 statistical software provided by Virginia Commonwealth University.

Scribble Antibody Creation

The C-terminus of zebrafish SCRIB was fused to GST and expressed in bacteria and purified using glutathione-sepharose beads. Purified GST-SCRIB protein was injected into mice. Whole blood was tested for the presence of reactive antibodies by immunostaining and western blot. Spleens were harvested, fused, and single B cell clones arrayed in 96 well plates. Individual clones were again tested for highly reactive antibodies by immunostaining and western blot analysis. The staining conducted in this screen was from supernatant collected from clone P2H4-D12-G5-E2, which is an IgG2 antibody.

Western Blotting

Embryos at 24hpf were dechorionated with Pronase, then deyolked in 1/2 Ringer's solution. 1uL of nonidet-P40 lysis buffer per embryo was used and embryos lysed, rocked for 30mins at 4ºC, and centrifuged for 20 mins at 18.8xg. SDS sample buffer with 10% β-mercarptoethanol was added at 1:4 concentration and boiled for 10 mins, then placed on ice for 2 minutes. Pre-cast MINI-PROTEAN® protein gels were used and between 90µg-100µg of protein was loaded into each lane. Western immunoblotting was conducted on transfer membrane after 5% BSA block for at least 1 hour. Scrib antibody was used at 1:10 concentration with 5% BSA overnight and subsequently washed with 1 x PBS 3x5mins. Secondary LI-COR® IRDye 680RD

Goat anti-Mouse IgG (H+L) was used at 1:5000 concentration with 5% BSA for at least 2 hours and subsequently washed with 1 x PBS 3x5mins. Blots were imaged with LI-COR® Odyssey (Virginia Commonwealth University).

FBMN-FBMN Puncta Quantification

Puncta along membranes of FBMN-FBMN contact were counted by located membranes of contact. The beginning and end of membrane contact along the Z-axis was determined visually utilizing Zen Blue. The length of the contact membrane was measured and recorded and all puncta counted by hand, through the Z-axis range. For normalization, puncta number along a specific membrane were divided by the length of the membrane on which those puncta existed. Student t-test was utilized to determine significance. All statistical tests were conducted with JMP11 statistical software provided by Virginia Commonwealth University.

Figures

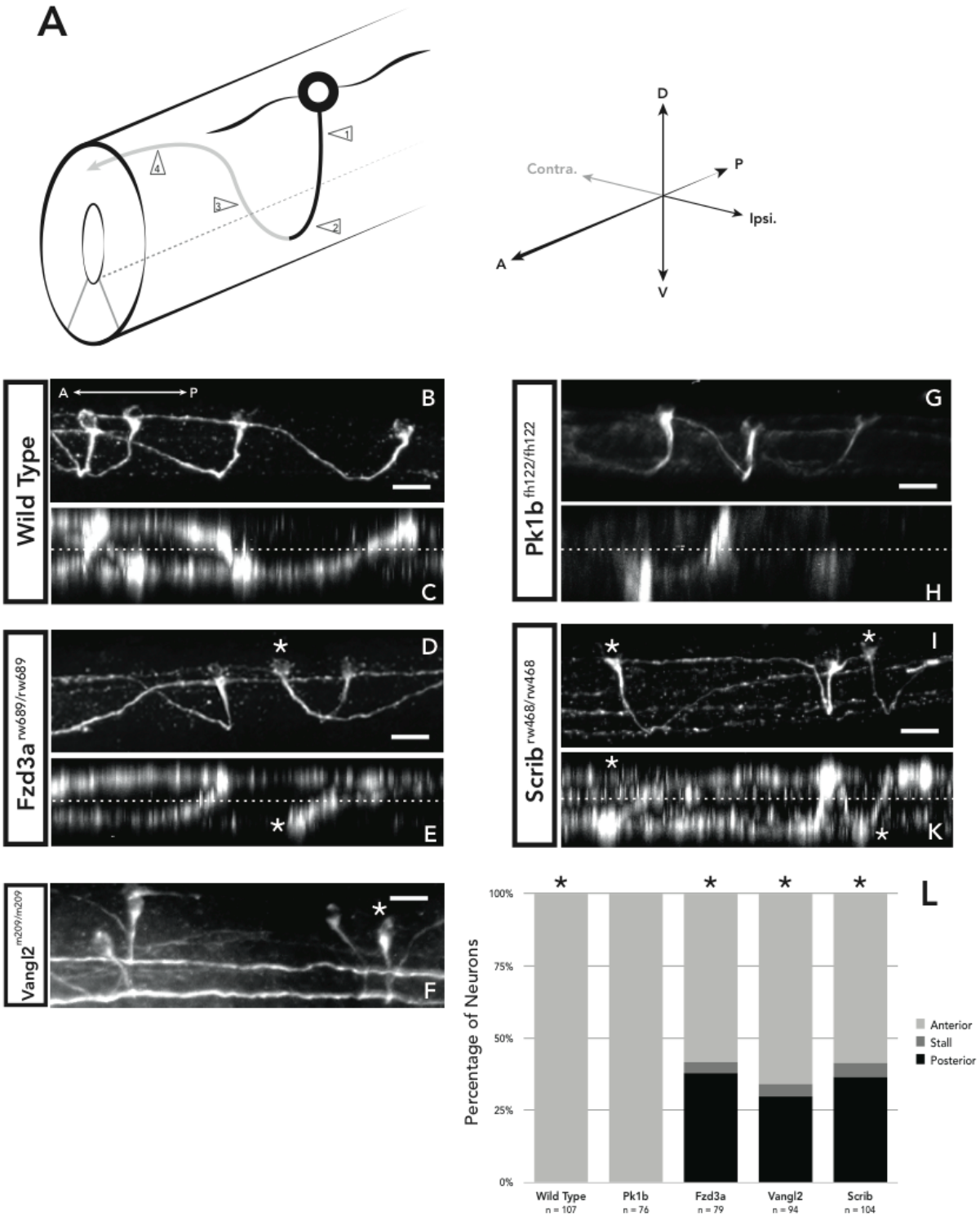

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Figure 1: CoPA axon pathfinding requires PCP components Fzd3a, Vangl2, and Scrib. At 33hpf, CoPA axon pathfinding is complete. **(A)** Schematic representing a single CoPA neuron with axial legend. Numbered arrowheads indicate steps of typical CoPA axon pathfinding (1) ipsilateral ventral projection (2) midline crossing (3) dorso- (4) anterior projection. **(B, D, F, G, I)** Confocal maximum intensity projections of 3A10 immunofluorescence in the spinal cord of **(B)** wild type, **(D)** *fzd3a*$^{rw689/rw689}$ **(F)** *vangl2*$^{m209/m209}$ **(G)** *pk1b*$^{fh122/fh122}$ and **(I)** *scrib*$^{rw468/rw468}$. Asterisks indicate example CoPA neurons that are pathfinding incorrectly posterior. **(C, E, H, K)** Confocal orthogonal projections of 3A10 immunofluorescence in the spinal cord of **(C)** wild type, **(E)** *frd3a*$^{rw689/rw689}$ **(H)** *pk1b*$^{fh122/fh122}$ and **(K)** *scrib*$^{rw468/rw468}$. White line approximates midline of embryo. In all images, anterior is to the left and posterior is to the right unless otherwise noted. **(L)** Percentage comparisons of CoPA axon projections after crossing the midline of all mentioned genotypes. Scale bar = 20μm. * $p < 0.0001$, Pearson's Chi-Square Test.

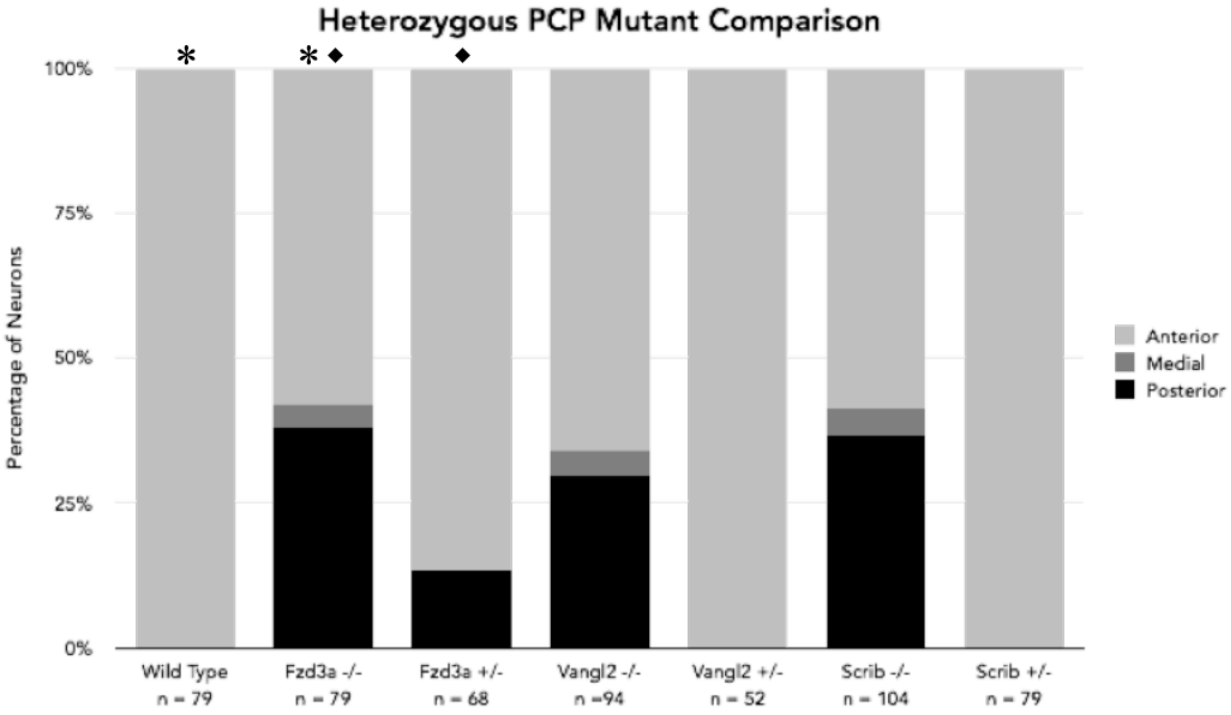

Figure 2: Fzd3a heterozygotes have a mild CoPA pathfinding phenotype. Percentage comparisons of CoPA axon projections after crossing the midline with heterozygous mutants for PCP components. In *fzd3a*$^{+/rw689}$ embryos, 15.3% of CoPA axons project posteriorly. * $p < 0.0001$, ◆ $p < 0.01$

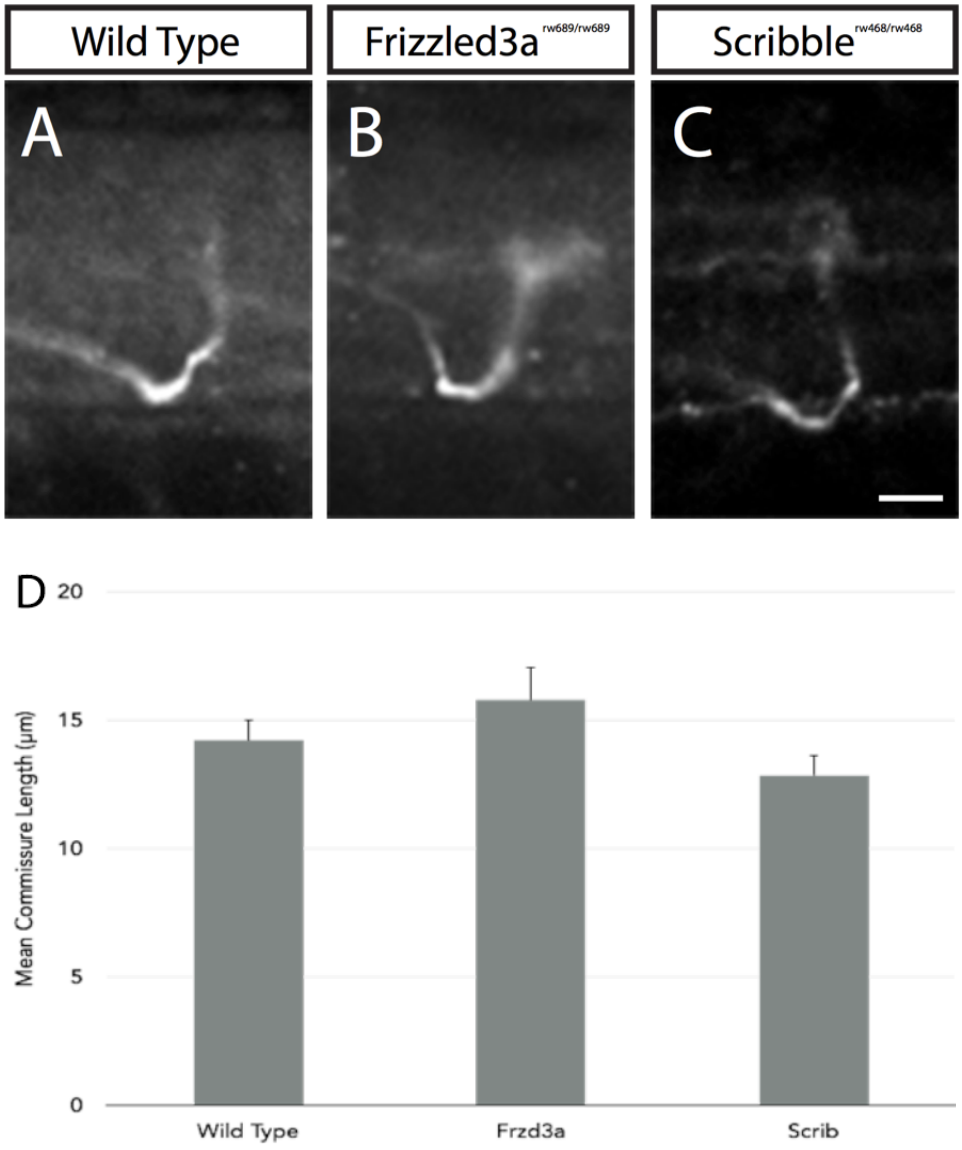

Figure 3: Anatomy and patterning of CoPA commissures remain intact in PCP mutants. **(A-C)** Single confocal planes of 3A10 immunofluorescence in the spinal cord of 33hpf embryos. **(A)** wild type CoPAs, **(B)** *fzd3a*$^{rw689/689}$ CoPAs, and **(C)** *scrib*$^{rw468/rw468}$ CoPAs. Scale bar = 10μm. **(D)** Mean commissure length of measured CoPAs. No significant difference was determined between genotypes (WT-fzd3a $p=0.26$; WT-scrib $p=0.27$; Student's t-test). Error indicates SEM.

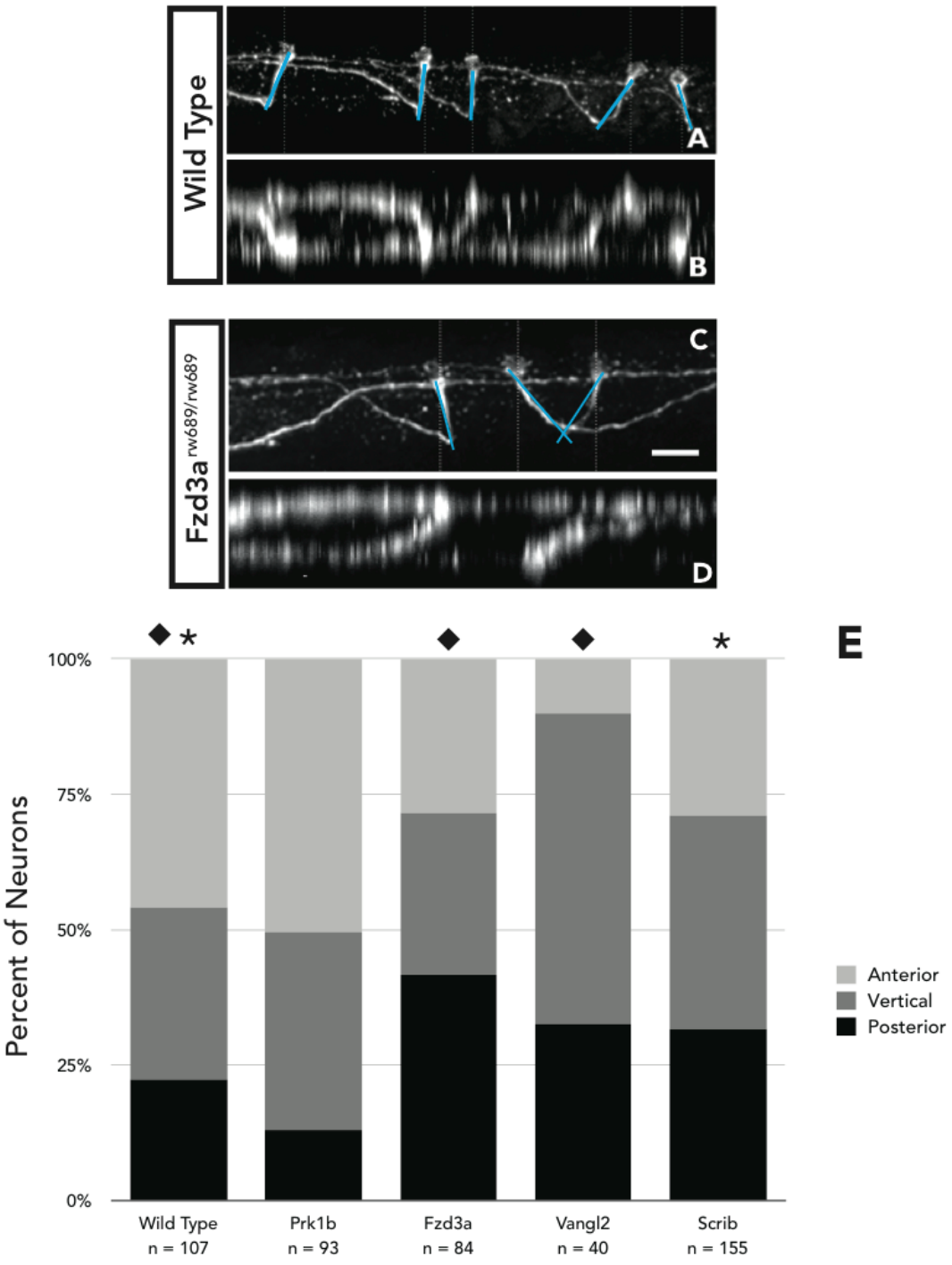

Figure 4: PCP components influence CoPA axons before midline crossing. (A & C) Confocal projections of 3A10 immunofluorescence in the spinal cord of **(A)** wild type and **(C)** *fzd3a*$^{rw689/rw689}$ mutants at 33hpf as examples of the method of measurement of pre-midline crossing fibers. **(B & D)** Confocal orthogonal projections of 3A10 immunofluorescence in the spinal cord of **(B)** wild type and **(D)** *fzd3a*$^{rw689/rw689}$ mutants at 33hpf. **(E)** Distribution of the direction of pre-midline axons within genotypes. * $p < 0.01$, ◆ $p < 0.05$; Pearson chi square test.

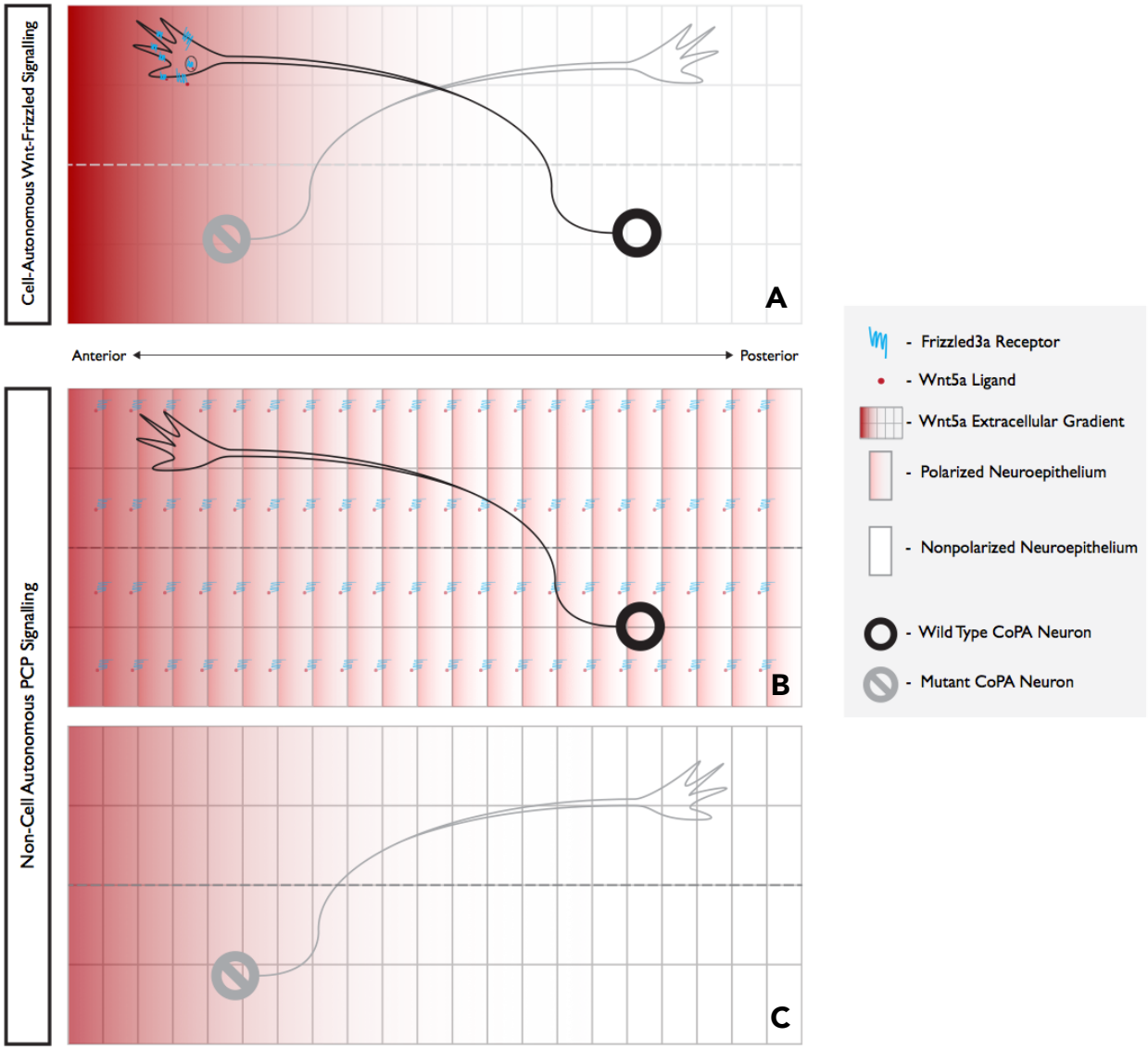

Figure 5: Two models for PCP-guided anterior-posterior CoPA Pathfinding. Two non-mutually exclusive models, **(A)** Cell-autonomous Wnt-Frizzled signaling and **(B & C)** Non-cell autonomous PCP signaling. In cell-autonomous model **(A)** axons are utilizing Fzd3a receptor to respond to extracellular Wnt5a gradient. In *fzd3a*$^{rw689/rw689}$ mutants, CoPA neurons cannot capability to respond to Wnt5a gradient and pathfind incorrectly. In non-cell-autonomous model **(B & C)** axons are responding to a neuroepithelium that has been polarized along the anterior-posterior axis. In a PCP mutant context **(C)** the neuroepithelium is unable to polarize, and as a result, the CoPA neuron pathfinds incorrectly.

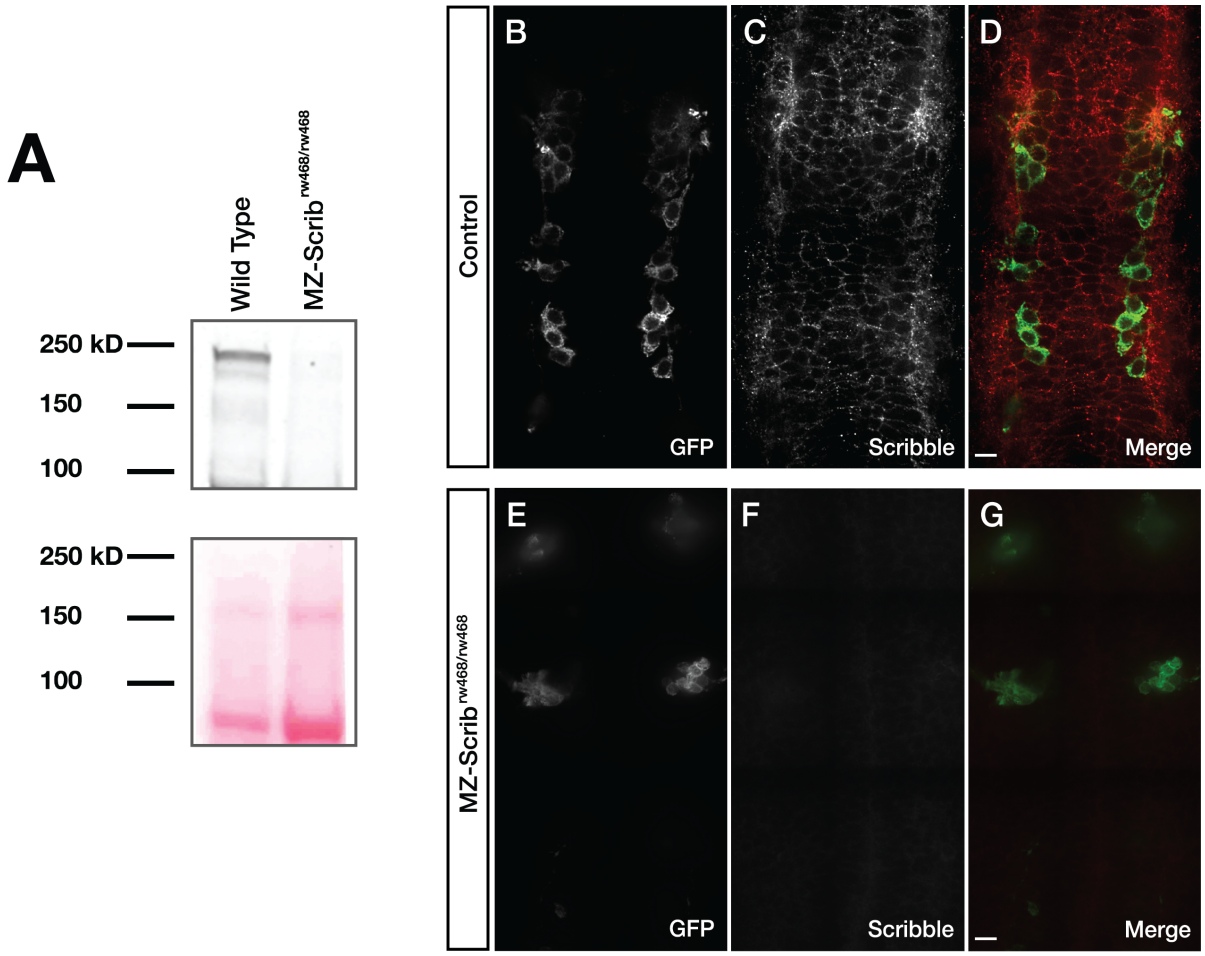

Figure 6: Monoclonal antibody is specific against Scrib. (A) Western blot utilizing our IgG2b monoclonal mouse antibody against the C-terminus of zebrafish Scrib and ponceau loading control. Lane 1 is total protein from wild type embryos and lane 2 is total protein from MZ-*scrib*$^{rw468/rw468}$. Note the absence of a distinct Scrib band in MZ-*scrib*$^{rw468/rw468}$. **(B-D)** Immunocytochemistry of **(B-D)** wild type isl1:GFP control neuroepithelium and FBMNs at 24hpf and **(E-G)** of MZ-*scrib*$^{rw468/rw468}$ embryo. Note the absence of signal in **(F)** while GFP staining in the FBMNs is still present in MZ-*scrib*$^{rw468/rw468}$ embryo **(E, G).** Scale bar = 10μm.

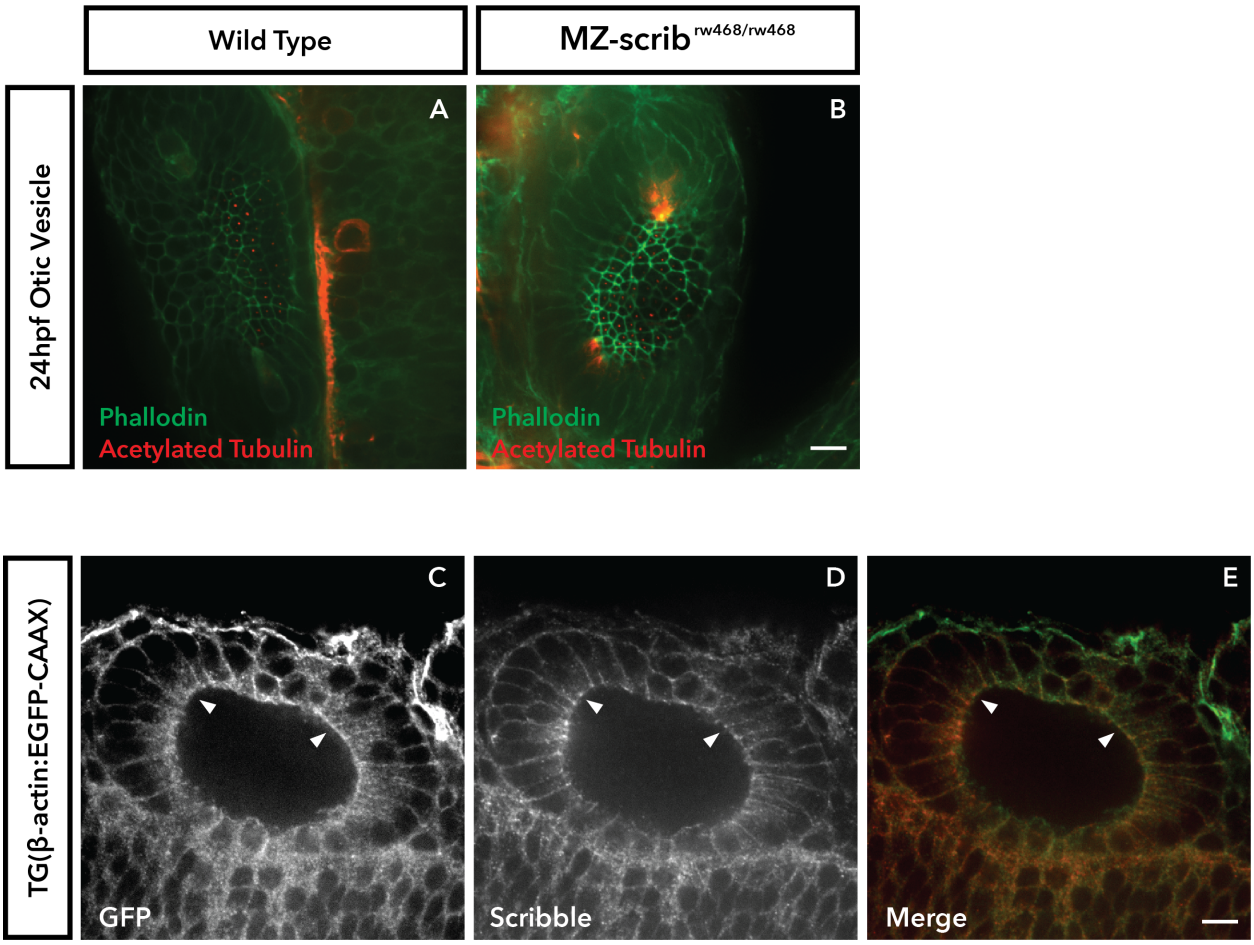

Figure 7: Scrib localizes to basolateral membrane in vertebrate epithelium. (A-B) Single confocal planes of F-actin (phallodin) and acetylated tubulin staining of **(A)** wild type and **(B)** MZ-*scrib*$^{rw468/rw468}$ embryos of the otic vesicle. Note the central location of otic cilia (acetylated tubulin) staining in **(A)** and **(B)**. **(C-E)** Single confocal planes of **(C)** GFP**,** and **(D)** Scribble immunocytochemistry of otic vesicle in Tg(β-actin:EGFP-CAAX) embryos at 24hpf. Arrowheads indicate examples of distinct puncta at the AB boundary. Scale bar = 10μm.

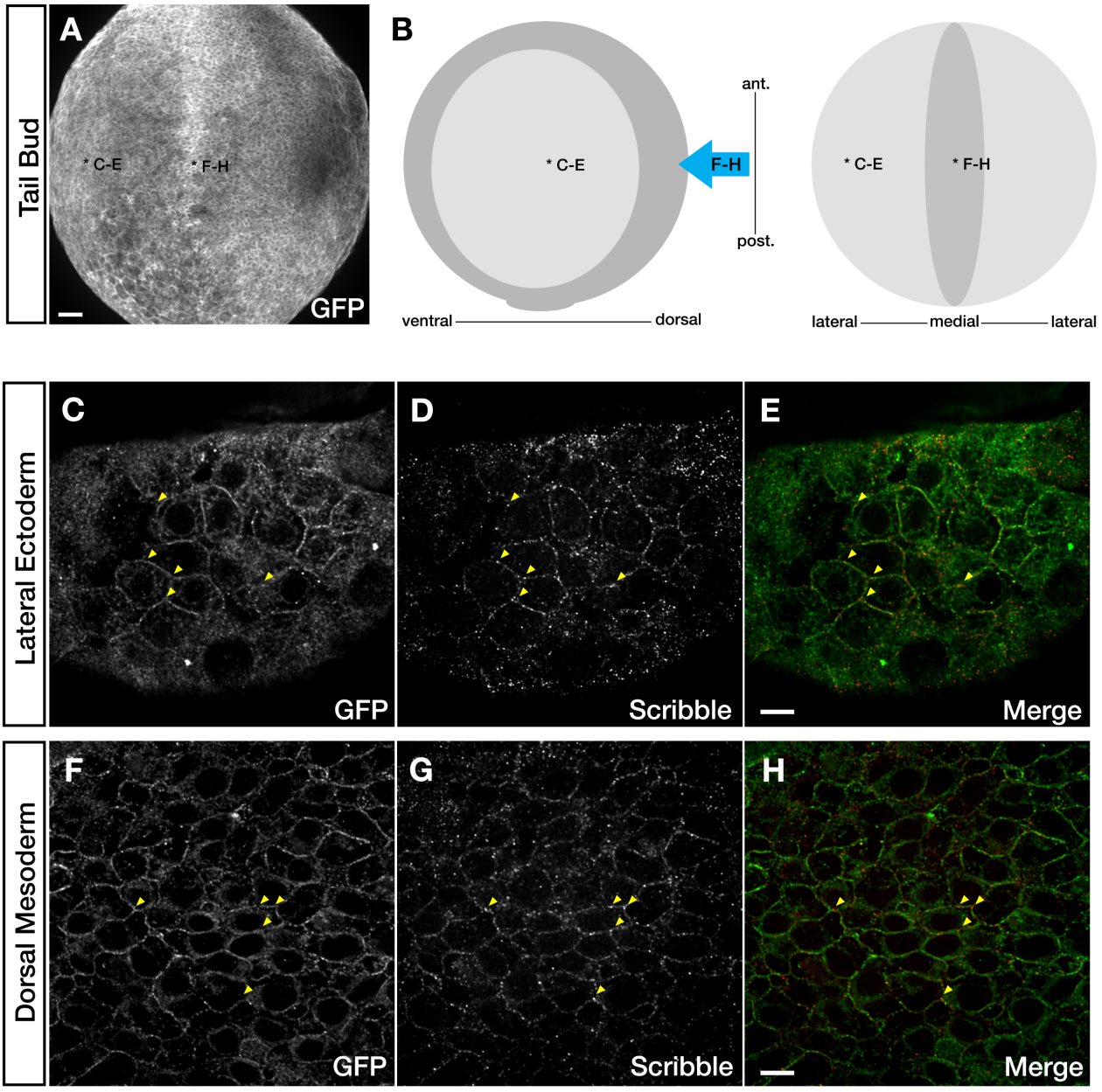

Figure 8: Scrib localized to the membrane and as puncta during CE movements. Immunocytochemistry for **(A, C, F)** GFP and **(D, G)** Scrib in Tg(β-actin:EGFP-CAAX) embryos at Tail Bud stage. **(B)** Schematic representation of perspectives presented at high magnification of the **(C-E)** Lateral Ectoderm and the **(F-H)** Dorsal Mesoderm. Yellow arrowheads indicate distinct Scrib puncta localized to membranes of cells. Scale bar (A) = 50μm, Scale bar (C-H) = 10μm

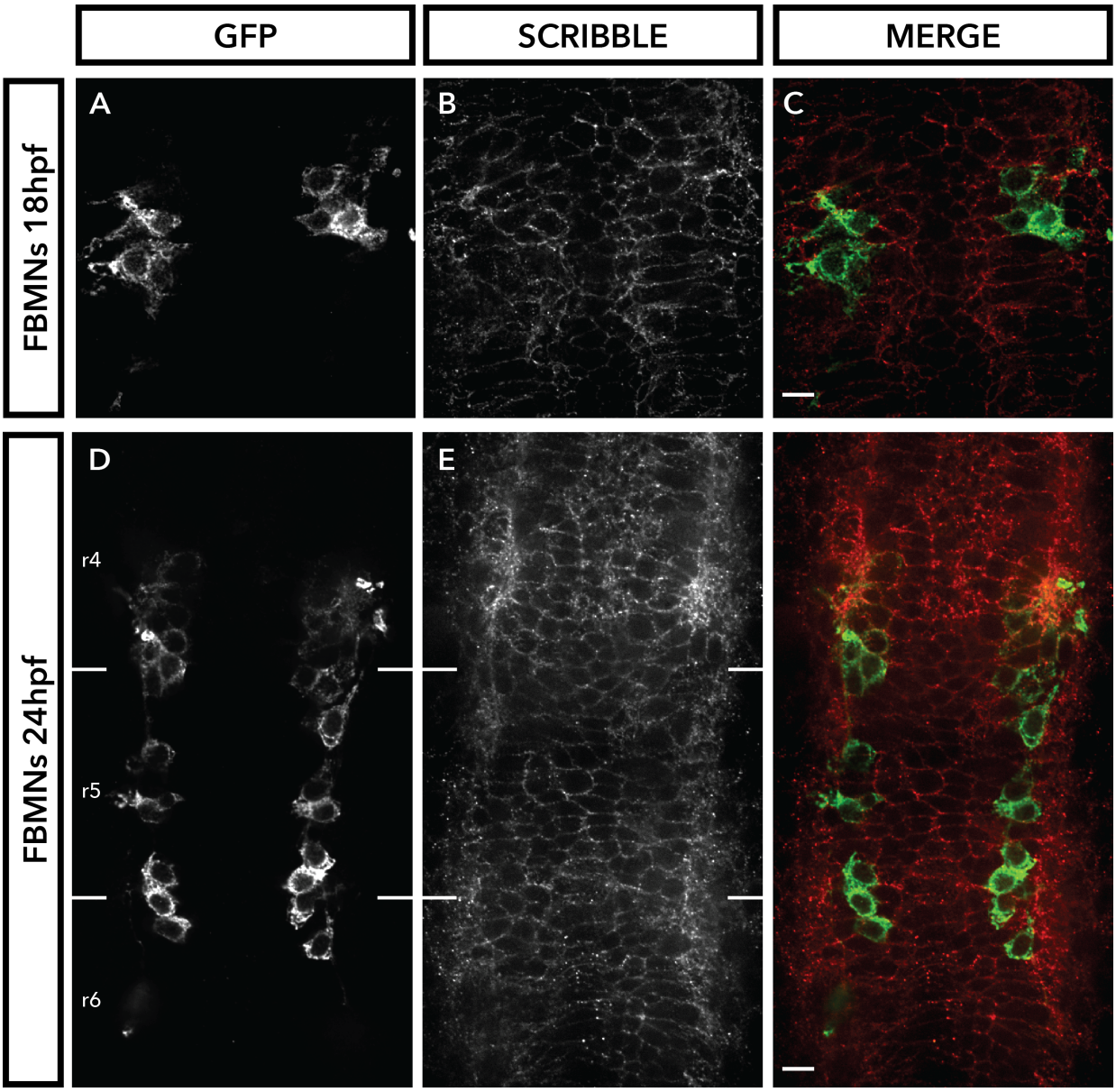

Figure 9: Scrib in migrating FBMNs localizes to membranes and as distinct puncta. Single confocal planes of migrating FBMNs at **(A-C)** 18hpf and **(D-F)** 24hpf. At 18hpf **(A)**, FBMNs are born and beginning to migrate. At 24hpf FBMNs **(D)**, are migrating through r5 into r6. Scale bar = 10μm.

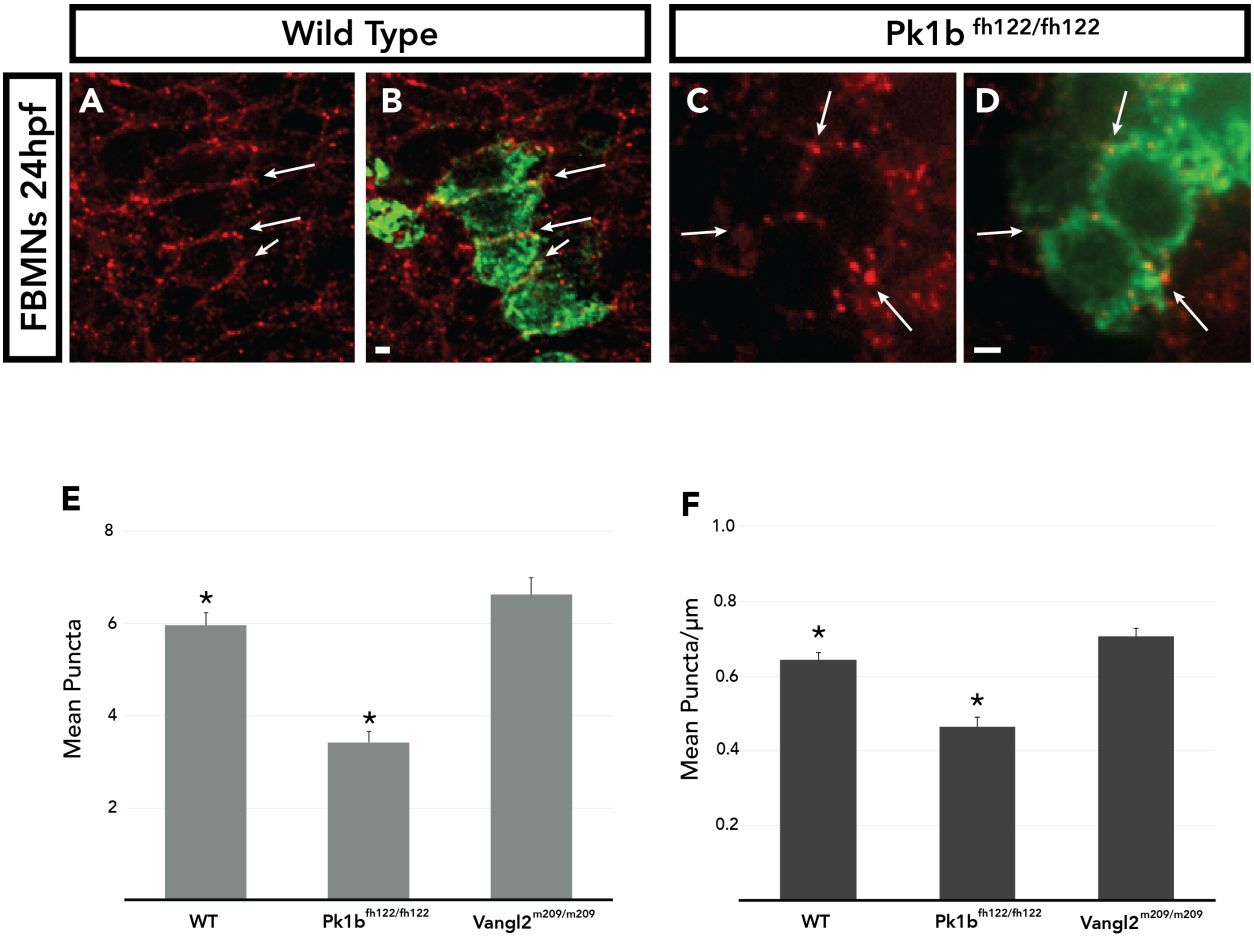

Figure 10: Scrib puncta distribution along sites of FBMN-FBMN contacts is influenced by Prk1b. (A-D) Single confocal planes as examples of puncta analysis along membranes of FBMN-FBMN contacts of **(A,B)** wild type embryos and **(C,D)** *pk1b*$^{fh122/fh122}$ embryos at 24hpf, during FBMN migration. Arrows indicate membranes along which puncta were counted. **(E,F)** Quantification of Scrib puncta by **(E)** mean counted puncta and by **(F)** normalization by length of the membrane along which the puncta were counted. * $p < 0.0001$, Student's t-test, error bars signify SEM. Scale bars = 2000nm.

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Supplementary Materials

Mammal	Chick	Xenopus tropicalis	Zebrafish
CELSR1	+	+	celsr1a, celsr1b
CELSR2	-	+	+
CELSR3	+	+	+
FZD3	+	+	fzd3a, fzd3b
FZD6	+	+	+
VANGL1	+	+	+
VANGL2	+	+	+
DVL1	+	+	dvl1a, dvl1b
DVL2	-	+	+
DVL3	+	+	+
PRICKLE1	+	+	prk1a, prk1b
PRICKLE2	+	+	+
PRICKLE3	-	+	+
PRICKLE4	-	+	-

Table S1: Core PCP genes in vertebrates. Genes that are present in *Ensembl* database as of Spring 2013. Zebrafish possess paralogous genes, designated ‘a’ and ‘b’ due to partial tetraploidy. Adapted from review Tissir, F. & Goffinet A., 2013.

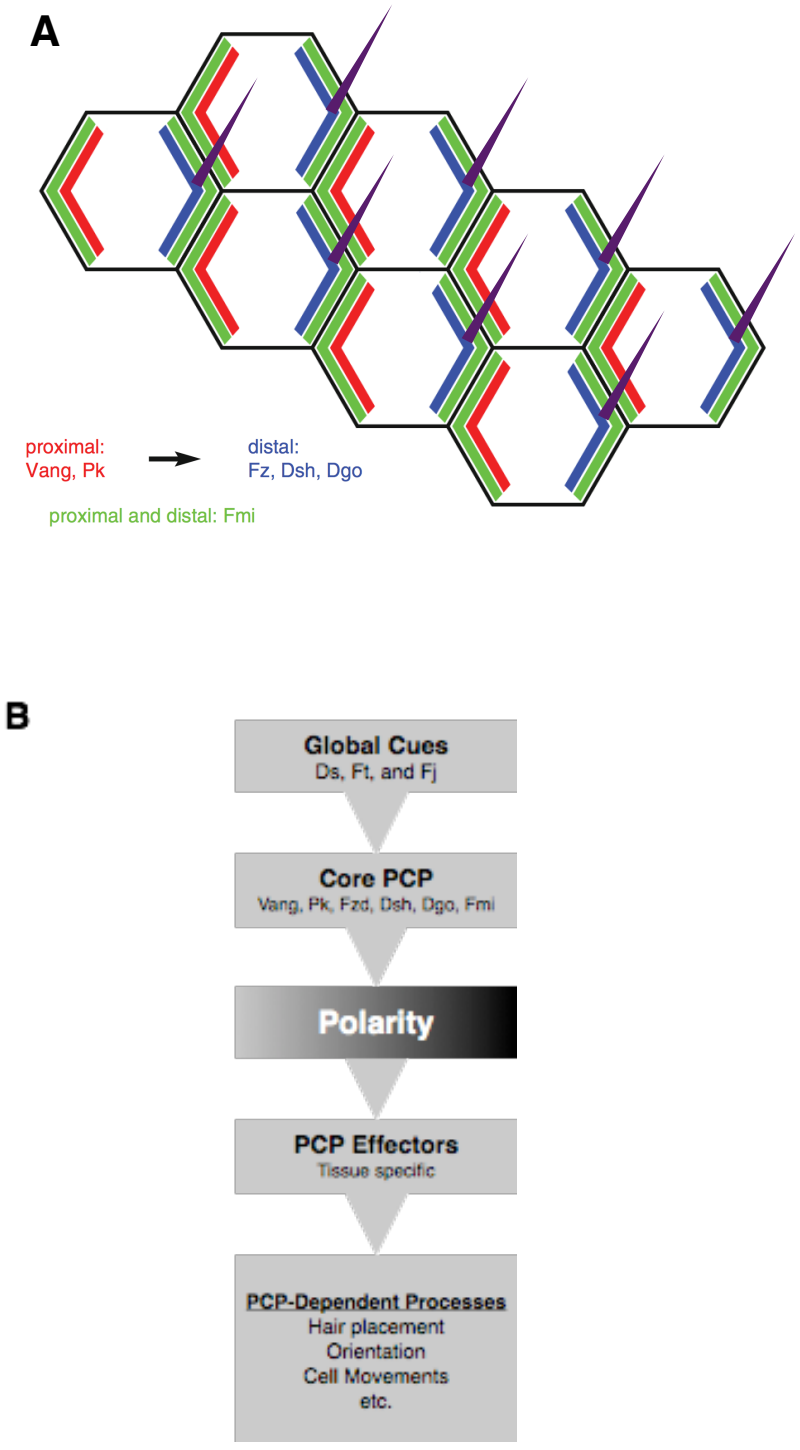

Figure S1: PCP model. (A) Core polarity proteins asymmetrically localize in the apical zone of *Drosophila* wing epithelium, translated to the placement of distal actin-based hair (represented by the purple triangle). Proximal is left, distal is right (adapted and modified from Vladar et al., 2009). **(B)** Model of non-canonical Wnt/PCP signaling and the proteins involved.

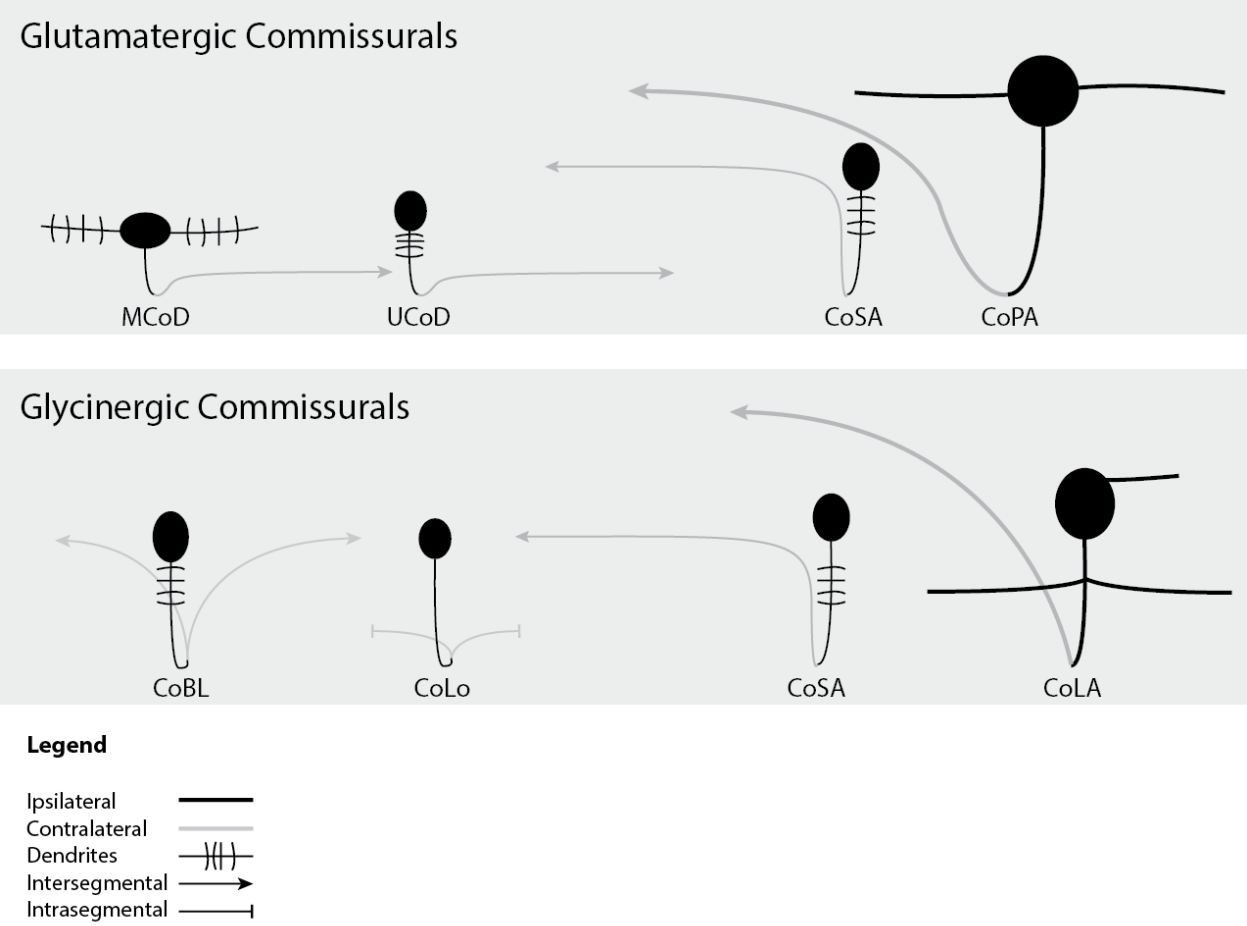

Figure S2: Commissural neurons of the zebrafish spinal cord. Neurons of the zebrafish spinal cord that cross the midline, divided by neurotransmitter classes: glutamatergic, glycinergic. Note the distinguishing phenotypes between the CoPAs and CoSAs. Adapted from McLean et al., 2007; compiled from Bernhardt et al., 1990; Kuwada et al., 1990a; Kuwada et al., 1990b; Hale et al., 2001; Higashijima et al., 2004.e

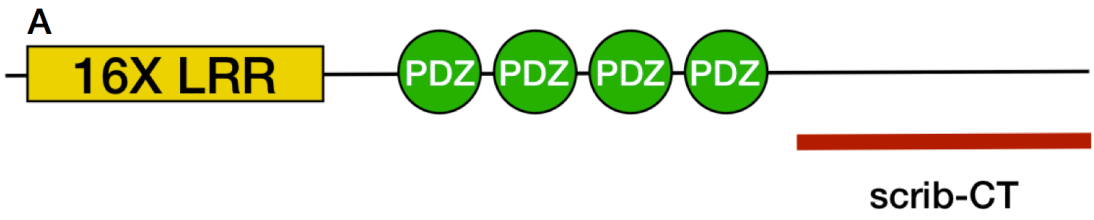

Figure S3: Model of protein structure of Scribble. Scribble begins with 16 leucine rich repeats and is followed by four PDZ domains. The antibody for zebrafish Scribble was against the C-Terminus, with the region injected into mice signified by the red marker.

Vita

Simon Dow-Kuang Sun was born May 25th, 1989 in Washington, DC to Paul and Louisa Sun. He received his Bachelor's of Science in Neuroscience and Music from the College of William & Mary in Williamsburg, VA in May of 2011. He will receive his Masters of Science in Biology at Virginia Commonwealth University and was awarded as the Outstanding Graduate Student in Cellular & Molecular Biology. This work was completed under the Thesis & Dissertation Award from the VCU Graduate School of Arts & Sciences. He will attend the Neural Science Doctoral Training Program at New York University beginning in the Fall of 2014.