Roles of Planar Cell Polarity Proteins in CoPA Axon Pathfinding

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ROLES OF PLANAR CELL POLARITY PROTEINS IN COPA AXON PATHFINDING

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

by

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List of Abbreviations

AB-Apicobasal

A-P: Anterior-posterior

CE: Convergent extension

Celsr: Cadherin EGF LAG seven-pass G-type receptor

CiD: Circumferential descending

CoBL: Commissural bifurcating longitudinal

CoLA: Commissural longitudinal ascending

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: Daschous

Dsh, Dvl: Disheveled

FBMN: Facial branchiomotor neuron

Fmi: Flamingo

Fj: Four-jointed

Fzd: Frizzled

Gpc: Glypican

GFP: Green fluorescent protein

IC: Ipsilaterally projecting interneurons

JNK: Jun-N-terminal kinase

MCoD: Multipolar commissural descending

MZ: Maternal zygotic

NTD: Neural tube defect

PBS: Phosphate-buffered saline

PBSTx: Phosphate-buffered saline Triton-X 100
PCP: Planar cell polarity

Pk: Prickle

Ptk7: Protein kinase 7

R4/r6: Rhombomeres 4 and 6

Scrib: Scribble

UCoD: Unipolar commissural descending

Vangl: Van Gogh like

Wnt: Wingless-type MMTV (mouse mammary tumor virus) integration site
Abstract

ROLES OF PLANAR CELL POLARITY PROTEINS IN COPA AXON PATHFINDING

By Ashley Morgan Purdy, B.S.

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

Virginia Commonwealth University, 2016
Major Advisor: Gregory S. Walsh, Ph.D.
Associate Professor, Department of Biology

In zebrafish, CoPA (Commissural Primary Ascending) is the first among ascending commissural axons to pathfind anteriorly and form the spinal commissure. One pathway that guides their anterior growth is the planar cell polarity (PCP) signaling pathway, but it is not fully known how PCP signaling regulates anterior guidance. We examined CoPA pathfinding in various PCP mutants to determine if anterior-posterior (A-P) guidance of CoPAs is dependent on PCP signaling. We found that certain PCP mutants exhibited anterior pathfinding defects, with approximately half of all affected CoPAs migrating incorrectly posteriorly. By using a translation-blocking DCC (Deleted in Colorectal Cancer) morpholino to prevent CoPA midline crossing, we discovered that CoPA axons in Fzd3a and Scribble mutants show severe defects in A-P guidance, which suggest that PCP influences A-P guidance of CoPAs prior to and after midline crossing.
Axon Guidance Mechanisms

Axon pathfinding is a fundamental feature of building neural circuits that control behavior. One-hundred billion neurons in the adult brain must form synapses with target cells, with the wiring of these neural circuits that compose the brain being no small task, as precise patterning is essential for the proper functioning of the nervous system. It is of interest to study the formation of synapses and the guiding of axons in the absence of injury in order to better understand how to address the problems of failure of axon regeneration and axon repair following spinal cord injury. Specifically of interest is studying chemoattractive cues that guide axons to their proper targets. Axons are guided by at least four different mechanisms: secreted chemoattractants and secreted chemorepellants, as well as contact-mediated attractants and contact-mediated repellants, which can act as short or long range cues depending on the cell context. In order to find their way in a complicated extracellular environment, axons are equipped with growth cones, highly sensitive, motile structures composed of a dynamic actin cytoskeleton that allows them to sense their environment at the tip of the growing axon. Surprisingly, there are only a handful of known molecules that are essential for this amazing feat of nervous system patterning. These are highly conserved and well-understood axon guidance molecules that include four families: the Netrins, Slits, Semaphorins, and Ephrins (Tessier-Lavigne and Goodman, 1996). More recent studies have added to this list a number of classic morphogens that were best
known for their role in regulating cell fate and differentiation, but are now known to control neuron migration and axon pathfinding. These include Bone morphogenetic proteins (BMPs), Wnts, Sonic hedgehog (SHH). Axons use these guidance cues to respond to dorso-ventral (D-V) and anterior-posterior (A-P) guidance cues in a variety of different cell contexts. Recent evidence has suggested that guidance of some axons along the anterior-posterior axis of the spinal cord may be controlled by Wnt-mediated activation of the planar cell polarity (PCP) pathway (Shafer et al., 2011).

**Planar Cell Polarity (PCP)**

Planar cell polarity (PCP) refers to the coordinated alignment of cells within the plane of an epithelium. It involves the core PCP proteins, Frizzled (Fzd), Celsr, Van Gogh-like (Vangl), Disheveled (Dsh), Prickle (Pk), and Scribble (Scrib), which are evolutionarily conserved from invertebrates to mammals (Figure 1). Each core PCP gene possesses a vertebrate orthologue (Tissir et al., 2013). The function of the core PCP proteins comes mostly from the study of epithelial polarity in the wing of *Drosophila*. Wing epithelial cells are hexagonally packed and contain an actin-based hair called a trichome that is asymmetrically localized to the distal side of the epithelial cells. The asymmetric distribution of these core proteins establishes polarity within the plane of the epithelium, perpendicular to apicobasal polarity (A-B), with Fzd, Dsh, and Diego colocalized to the distal side and Vangl and Pk colocalized to the opposite proximal side of the cell. This asymmetric
distribution of PCP components leads to morphological polarization through the regulation of the cytoskeleton, in this case the polymerization of actin for trichome formation. It is now established that PCP plays an evolutionarily conserved role in epithelial polarity in vertebrates, regulating the planar orientation of hair follicles, inner ear hair cells, monociliated positioning in node cells and floorplate cells, and kidney tubules.

Accumulating evidence suggests that PCP signaling shapes the developing nervous system by controlling several important events, such as, the establishment of polarity of primary cilia in the inner ear, the closure of the neural tube during neurulation, and cell intercalation during convergent extension in gastrulation and neurulation (Wallingford 2012). While previously thought to be distinct but complementary, an intimate relationship exists between Planar Cell Polarity and Apico-Basal Polarity (A-BP) that allows axon growth cones to navigate with highly specific sensitivity. New discoveries about PCP proteins are being made with increasing frequency, notably in the processes of neuronal migration, dendritic tiling, and axonal guidance, advancing PCP to an umbrella term, which is now operationally defined as, “any event that affects cell polarity and involves one or more of the core PCP genes”(Wallingford 2012).

PCP is thought to regulate cellular decisions along the anterior-posterior axes. Specifically, facial branchiomotor neurons (FBMN) use PCP proteins to migrate caudally from rhombomere 4 in the hindbrain to their final location in
rhombomere 6. Without PCP proteins, these FBMNs stall in r4 and never reach their final location in r6 (Bingham et al., 2002; Wada et al., 2005; Wada et al., 2006; Mapp et al., 2011; Walsh et al., 2011). The logical question that arises is, “Do other cell types in the nervous system depend on PCP signals to make the correct pathfinding decisions?”

**CoPA System and Pathfinding**

A commissural primary ascending (CoPA) neuron, is a T-shaped glutamatergic interneuron located dorsally in the spinal cord; its axon travels ventrally to cross the midline and then projects dorsally and anteriorly (Hale et al., 2001). CoPA neurons are the second order sensory neuron in a circuit involved in touch-evoked coiling responses in zebrafish (Pietri et al., 2009). Sensory neurons in fish, called Rohon-Beard neurons synapse on CoPA cells. These cells relay sensory information to the contralateral hindbrain where they synapse on descending motor interneurons, which relay information ipsilaterally to primary motoneurons to evoke contraction of muscles on the side of the embryo opposite the touch stimulus. The benefit of using the CoPA as a model to study axonal migration is that it serves as a pioneer axon that projects singularly, rather than in a bundle of cells, as seen in other model systems, such as mice (Lyuksyutova et al., 2003; Shafer et al., 2011). The CoPA is the first axon among ascending commissural axons to establish the commissure. This enables researchers to view axon pathfinding mechanisms on a single-cell level using CoPAs in zebrafish.
It is known that the growth cones of CoPAs use chemoattractant and chemorepellant cues to guide their axon pathfinding decisions, but it is not fully known how they utilize PCP proteins to aid their pathfinding. Our research aim is to test the hypothesis that CoPAs are using the PCP to guide anterior turning decisions. In order to do this, we first must understand how CoPAs pathfind normally, in the presence of PCP.

**PCP and Deleted in Colorectal Cancer (DCC) signaling in CoPAs**

In order to navigate the midline and project its axon to the correct location, a CoPA neuron’s growth cone must simultaneously respond and be insensitive to several chemoattractive and chemorepellant cues. Midline crossing occurs in a series of steps with the axon first extending ventrally at 17 hpf (hours post fertilization) in response to DCC-Netrin signaling, while being insensitive to Robo-Slit signaling. As the growth cone enters the floorplate, it begins to become insensitive to the attractant, Netrin, while simultaneously becoming sensitive to the midline repellant Slits. Passage and exit from the midline is dependent on the function of two Slit receptors, Robo2 and Robo3. After midline exit, Robo2, expressed by CoPA axons, is thought to continue to repel axons away from the midline, directing their growth to the contralateral dorsal spinal cord. Finally, the CoPA ascends and joins later born commissural neurons in the DLF (Dorsal Longitudinal Fasciculus) (Bonner et al. 2012).
Accumulating evidence indicates that planar cell polarity components are required for the anterior turning of commissural axons after midline crossing. In mice, three core membrane PCP proteins, Fzd3, Vangl2, and Celsr3 are essential for anterior guidance of commissural axons. Analysis of dorsal-most populations of commissural axons in the spinal cord, which extend as tracts of axons toward the midline, are incapable of sensing rostrocaudal guidance cues until after crossing the midline (Lyuksyutova et al., 2003; Shafer et al., 2011).

Another cue that guides commissural axons are Wnt ligands, secreted glycoproteins expressed in an anterior-posterior gradient in ventral midline cells of the floor plate. Wnt binding to the Fzd3 receptor has been shown to activate the Jun-n Terminal Kinase pathway (JNK) in the spinal cord (Zou et al., 2012). Open-book spinal cord explant experiments in mice have shown that exogenously applied Wnts cause growth cones to enlarge and turn anteriorly towards the Wnt gradient after midline crossing (Lyuksyutova et al., 2003). More recent experiments have linked PCP proteins to controlling membrane localization of the Fzd3a receptor (Shafer et al., 2011). It is now known that the PCP proteins, Disheveled1 and Vangl2 have antagonistic functions on Fzd3a, with Dvl1 promoting plasma membrane localization of Fzd3a and Vangl2 promoting Fzd3a internalization (Shafer et al., 2011). Vangl2 activity serves as an indicator of PCP activity, as Vangl2 is highly enriched on the tips of growing growth cone filopodia (Zou et al., 2012). Furthermore, Dvl1 and Dvl2 have an antagonistic relationship
that enhances the existing PCP signal initiated by Vangl2. Dvl2 activates aPKC (atypical protein kinase C) and Dvl1 blocks Wnt/PCP signaling by keeping Fzd3a hyperphosphorylated on the plasma membrane (Onishi et al., 2013).

Experiments have been conducted in mice to determine if midline crossing is required for subsequent pathfinding decisions. Examination of Fzd3 knockout mice showed no defect in the pre-midline trajectory of commissural axons; the defect in anterior turning only becomes evident after midline crossing, although analysis of tracts of axons makes it difficult to discern subtle changes in axon trajectories (Lyuksyutova et al., 2003). It is clear that commissural axons are sensitive to exogenously applied Wnt ligands. In “open-book" spinal cord organotypic culture techniques, commissural axons will pathfind toward CV-1 origin SV40 (COS) cells that secrete exogenous Wnt ligands, but appear to only turn toward a source of Wnts after midline crossing (Zou et al., 2012). This supports the idea that commissural axons respond to guidance cues in a sequential manner, in that they must respond to one guidance cue before becoming sensitive to another cue. Arguing against that notion are experiments done in zebrafish looking at CoPA pathfinding, when midline crossing is inhibited by DCC knockout (Bonner et al., 2012). These authors demonstrated that midline crossing is not required, meaning that CoPAs can respond to anterior guidance cues without crossing the midline (Bonner et al., 2012).
PCP is required for CoPA Axon Pathfinding

Previously, the Walsh lab examined CoPA axon pathfinding in fzd3a, vangl2, and scribble mutants. All of the PCP mutants displayed a similar phenotype. In particular, we showed that Frizzled3a and Vangl2 have evolutionarily conserved roles in commissural growth cone steering along the anterior-posterior axis of the developing neural tube. Using loss-of-function PCP mutants, the Walsh lab showed that CoPA axons properly responded to D-V guidance cues and navigated the midline appropriately, but are misguided along the anterior-posterior axis. With single neuron resolution, we showed that loss of fzd3a or vangl2 or scribble genes leads to randomized growth of CoPA axons along the A-P axis. Interestingly, CoPA axons do not wander or stall, but grow and extend for long distance in either the anterior or posterior direction. Together, these data support two major conclusions, i) PCP proteins are not required for axon growth per se, but are essential for proper anterior turning of commissural axons, and ii) PCP proteins do not affect dorsal-ventral guidance decisions or midline crossing by commissural axons. We aim to identify additional PCP components involved in CoPA axon pathfinding, including possible Wnt-Fzd coreceptors, Protein Tyrosine Kinase 7(Ptk7) and Glypican 4(Gpc4), the transmembrane protein, EGF LAG 7pass G type receptor 3 (Celsr3), and downstream effectors, such as Jun-N Terminal Kinase (JNK). Finally we aim to determine the role of PCP prior to midline crossing and the role of a Wnt gradient in vivo. We have previously shown that
the anterior pathfinding in CoPA fibers requires, Fzd3a, Vangl2, and Scribble proteins. This suggests that commissural axon pathfinding is at the very least a PCP-like phenomenon, but it is unclear whether this process is dependent on the function of all PCP components in vertebrates. For instance, several membrane bound or transmembrane proteins have been implicated as Wnt-co-receptors in various cell contexts, including Glypican4 and Ptk7. We therefore sought to determine whether these PCP components are required for CoPA pathfinding.

**Other PCP components being tested:**

1) **Protein Tyrosine Kinase-7 (Ptk7)**

Protein tyrosine kinase-7 (Ptk-7), also known as colon carcinoma kinase 4 (CCK-4) has seven transmembrane domains and a catalytically inactive kinase domain, also called pseudokinase domain (Mossie et al., 1995). It was originally identified as upregulated in colon carcinoma and has since been revealed to be a novel regulator of PCP signaling with orthologues in mouse, Xenopus, chick (KLG), Drosophila (Off-track, OTK) and Hydra (Lemon) (Chou and Hayman, 1991; Lee et al., 1993; Mossie et al., 1995; Miller and Steele, 2000; Winberg et al., 2001; Jung et al., 2004). In mice, Ptk7 has been shown to genetically interact with Vangl2 (Lu et al., 2004). Also, mutations in mouse Ptk7 confer neural tube defects such as cranioschisis and failure of cochlear stereocilia to orient correctly (Lu et al., 2004). These Ptk7 mouse mutants exhibit the same severe neural tube defects as Vangl2-looptail mice, Celsr-1 mouse mutants, and Scrib-circletail mice (Lu et al.,
2004). Also, disruption of stereocilia bundle orientation is shared between Ptk7 mouse mutants and Vangl2 and Scrib mouse mutants (Lu et al. 2004). Similar phenotypes are observed in Xenopus, as Xptk7 MO-injected embryos exhibit convergence extension problems, and failure of neural tube closure (Lu et al., 2004). Ptk7 co-immunoprecipitates (co-IPs) with Wnt3a and Wnt8 in mice, which may require a Ptk7 and Fz7 receptor complex (Peradziryi et al., 2011), leading to the suggestion that Ptk7 may function at least in some cell contexts as a Wnt coreceptor. In zebrafish, Ptk7 has been shown to play a role in inhibiting exogenous Wnt8 activity (Hayes et al., 2013). There is the possibility that Ptk7 potentiates PCP signaling through Wnt5 activity, suggested by low expression of both Wnt5 and Ptk7 mRNA yielding strong convergence extension phenotypes in vivo (Hayes et al., 2013). Ptk7’s pseudokinase domain, which interacts with Dvl, Rack1 and B-catenin, is thought to be essential for Ptk7 activity in mice, despite its lack of kinase activity (Lu et al., 2004; Shnitsar and Borchers, 2008; Puppo et al., 2011). In zebrafish, the plasma membrane tethered Ptk7 extracellular domain was sufficient to rescue PCP morphogenesis defects (Ciruna et al., 2013). Their results in zebrafish show that Ptk7 acts as an important regulator of non-canonical Wnt/PCP signaling, in a manner that requires its extracellular domain. We aim to determine Ptk7’s role in axon guidance using CoPAs in zebrafish as a model system.
2) EGF LAG 7pass G type receptor 3 (Celsr3)

Celsr3 is an atypical seven-pass cadherin receptor and is one of three vertebrate homologues of Flamingo (Fmi) first identified in Drosophila to play a role in dendritic growth and PCP (Lu et al., 1999; Usui et al., 1999; Gao et al., 1999). In mice, Celsr3 was shown to be essential for anterior turning of commissural axons (Zhou et al. 2012). In zebrafish, Celsr3 is required for the formation of the proper synaptic circuitry in the retina (Lewis et al., 2011). The role of Celsr3 in CoPA pathfinding in zebrafish has yet to be established, however; it has been implicated in brain development in the following ways: Celsr3 suppresses neurite outgrowth in hippocampal neurons in mice (Shima et al., 2007), Celsr3 is important for proper axonal tract formation in mice (Tissir et al., 2005; Ying et al., 2009; Zhou et al., 2007; Zhou et al., 2008) and for correct interneuron migration in the mouse forebrain (Zhou et al., 2009). We aim to determine a role for Celsr 3 in axon guidance using CoPAs in zebrafish as a model.

3) Jun-n terminal Kinase (JNK)

Jun-n terminal kinase (JNK) is a part of JNK pathway, a mitogen activated protein kinase pathway. This transduces signals within cells in response to many different stimuli and have differing results depending on the cell type and location, including differentiation, proliferation and regulation of other pathways (Marshall et al., 1995). The small GTPases Rac and Cdc42 and RhoA have been implicated in
the JNK pathway in vertebrates to act upstream of JNK. Additional experiments in *Drosophila* showed RhoA mutants have abnormal eye and wing polarity and found that fzd, dsh, and RhoA genetically interact (Strutt et al., 1997). It was proposed that RhoA and JNK interact in the same pathway because RhoA and JNK mutants have similar embryonic phenotypes. Later experiments in Xenopus revealed that Wnt5 is capable of activating JNK signaling in cultured cells and that this activated JNK is able to control CE movements (Yamanaka et al., 2002). In mouse hindbrain explant experiments, facial branchiomotor neurons failed to migrate after treatment with JNK and Rock inhibitors (Vivancos et al., 2009). In mice, chemical treatment with SP600125, which blocks all three JNKs, led to A-P randomization of commissural axons in “open book” spinal cord explants. Also, after cultured commissural mouse axons were treated with Wnt5 exogenously, levels of endogenous phospho-jun, the phosphorylated and active form of c-jun were increased by four times the normal amount compared to controls (Shafer et al., 2011). One aim of this study is to determine whether JNK is a downstream effector of PCP in axon pathfinding.

4) **Glypican 4(Gpc 4)**

Heparin sulfate proteoglycans are composed of secreted or membrane-bound proteins to which several heparin sulfate polysaccharide chains are added. *Knypek*, a zebrafish homolog of the mammalian *glypican 4*, encodes a member of the glypican family of heparin sulfate proteoglycans (Solnica Krezel et al., 1996).
Glypican 4 is the most-studied zebrafish glypican, and several glypican 4 mutants have been made. The glypican 4 mutant kny<sup>f6/f6</sup> contains a premature stop codon (S247) in the middle of the protein resulting in complete loss of function. In zebrafish, knypek mutants have convergent extension defects but have normal lateral cell fates, which suggest knypek is acting in a PCP dependent manner. Knypek/silberblick double mutants missing Glypican 4 and Wnt 11 revealed an additive effect on the CE defect and these effects were not due to an increase in BMP activity (Topczewski et al., 2001). Experiments performed in C. elegans on Syndican1, the only heparin sulfate proteoglycan present in C. elegans displayed aberrant axonal pathfinding in interneurons in Syn1 mutants, with neurons normally staying ipsilateral in WTs crossing the midline in Syn1 mutants (Rhiner et al., 2005). We aim to determine a role of Glypican 4 in axon guidance using CoPAs in zebrafish as a model.

**Aims of Present Study**

There are several aims of this study. The first aim is to determine which components of the PCP pathway are involved in CoPA axon pathfinding. The second is to determine if pre-midline crossing fibers can respond to PCP cues. The third is to determine the role of Wnt in axon pathfinding in vivo.
Materials and Methods

Fish Strains and mutants

Zebrafish (*Danio rerio*) 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 matings of AB. The *fzd3a* mutant was originally described as *off-limits/off*<sup>rw689</sup> (Wada et al., 2006). The *vangl2/trilobite* mutant was originally described as *trm<sup>209</sup>* (Jessen et al., 2002). The *scrib* mutant was originally described as *landlocked/llk*<sup>rw468</sup> (Wada et al., 2005). The *pk1b* mutant was originally described as *pk1b<sup>fh122</sup>* (Mapp et al., 2011). The *ptk7* mutant was originally described as *ptk7<sup>hsc9</sup>* (Hayes et al., 2013). The *glypican4* (*gpc4*) mutant was originally described as *knypek/gpc4*<sup>fr6</sup> (Topczewski et al., 2001 Dev Cell 1:251-264.). *Tg(tbx16:GFP)* fish were obtained from the Lardelli lab (Wells et al., 2011).

Embryonic genotyping

*MZptk7* homozygous mutant embryos were genotyped by their convergent extension phenotype. This was confirmed with fin-clipping and embryonic genotyping, using the SfaN1 restriction enzyme, which cuts wildtypes at 2 bands at 500 bp and 100 bp, heterozygotes at 3 bands at 600, 500, and 100 bp, but does not cut mutant *ptk7* PCR product. *ptk7* homozygous mutant females were crossed with heterozygous males. These embryos were identifiable by their short and fat body types. *Knypek* homozygous mutant embryos were genotyped by their CE.
phenotype after incross of two heterozygous carriers. *fzd3a* homozygotes were separated based on their facial motor neuron migration (FBMN) block. Those embryos with a complete FBMN block with neurons in R4 were determined to be homozygous. The DdeI restriction enzyme was used to genotype Fzd3a fish. It cut mutants at 85 bp and 30 bp, leaving wildtypes with one band at 115 bp. *val<sup>b337/b337</sup>* fish were genotyped by using PVUII restriction enzyme, which cuts WT down to 2 fragments of approximately 100 and 120 bp, but does not cut the PCR product from the *val-* allele. Heterozygotes had all 3 bands at 100, 120, and 220 bp. Homozygous mutants had 1 band present at 100 bp.

**Morpholino Injection**

The day prior to microinjections, injection dishes were prepared with 1.2% agarose poured into a petri dish float mold to create triangular wells with a 45 degree angle to create a backstop for needle pressure. The night before the fish that are going to be mated were setup with the males and females separated in tanks with a divider that can be easily removed to allow mating in the morning. The injection needle was prepared to allow a bolus to be injected through the chorion of the embryo at the one cell stage. When not in use, the microinjection needle tip was kept in mineral oil dish in order to prevent the needle from drying out or becoming clogged. Embryos were collected after mating and were pipetted into wells to be injected. Embryos were kept in incubator to mature and were sorted to dispose of unfertilized embryos. The *celsr3* splice-blocking MO was injected (15ng) into 1-
cell stage tbx16:GFP embryos. The celsr3 splice-blocking MO has the following sequence: (CTCCCGTTACTGAACCTTACCAGTGA). 3ng of dcc translation-blocking morpholino (GATATCTCCAGTGACGCAGCCCAT; start codon complement underlined), or 5ng of fzd3a splice-blocking morpholino (CAATGTGAATTGGTTTACCTCCATG) was injected at the one-cell stage using an ASI MPPI-3 (Applied Scientific Instrumentation) pressure injector. For double morpholino injections, fzd3a and dcc MOs were co-injected on the same day. All morpholinos were obtained from Gene Tools, LLC.

**SP600125 Inhibitor Treatment**

SP600125 JNK inhibitor was used to treat 15 hpf embryos at 1μM concentration. The inhibitor was diluted in fish water and the embryos were bathed in inhibitor solution from 15-24 hpf then were washed in fresh fish water and placed in PTU (1-phenyl-2-thiourea) water.

**Immunocytochemistry and immunofluorescence**

PTU (1-phenyl-2-thiourea) was put on embryos at approximately 24 hpf in 0.003 % fish water to prevent pigmentation. The embryos were dechorionated manually. The embryos were fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) overnight at 4°C at 31 hpf, when COPA migration is nearly complete and 3A10 staining is strong and specific for CoPA neurons. The embryos were permeabilized by cold acetone treatment for 7 minutes at -20 °C, then washed in PBSTriton (PBSTx) 3 times for 5 minutes each on a rocker. Then they were
blocked with PBSTriton, 4% goat serum, 4 mg/ml bovine serum albumin (BSA) for an hour at room temperature. Then 3A10 primary antibody was added to the embryos diluted in block overnight at 4 °C to stain COPAs. The 3a10 primary antibody stains for a neurofilament associated protein present in CoPAs. The embryos were washed in successive 30 minute PBSTriton washes 5x. The secondary antibody, goat anti mouse 568 antibody, was added to the embryos diluted in block overnight at 4°C. The embryos were washed in PBSTriton 5x for 30 minutes each rocking. Next, the embryos were dehydrated through, 25%, 50%, and 75% glycerol/PBS solutions.

**Heatshocking**

*Tg (hsp70: Wnt5b: GFP \textsuperscript{w33})* embryos were heatshocked at 15 hpf when CoPA neurons are first being born. The embryos were heatshocked twice for 30 minutes each in a 37°C water bath as previously described in order to ubiquitously express Wnt5b in the embryo and disrupt the existing gradient (Moon et al., 2007).

**Confocal microscopy**

All images were taken using a Carl Zeiss spinning disc confocal microscope at 63X magnification. The embryos fixed in 4% PFA were deyolked and mounted laterally in 75% glycerol on a glass slide with a coverslip. Confocal projections were made from imaging the located CoPAs on one side of the spinal cord through to the CoPAs on the other side of the spinal cord. Maximum intensity projections were created with Zen Blue software in order to visualize the CoPAs more clearly.
For live-imaging of CoPAs, embryos anesthetized with Tricaine then suspended in a glass-bottom dish surrounded with fish water.

Results

Ptk7 is required for proper CoPA axon pathfinding

We extended our findings by testing the role of Ptk7 in anterior guidance of CoPA fibers. First, we analyzed CoPA pathfinding in zygotic ptk7^{hsc9/hsc9} fish. We found, as first reported by the Ciruna Lab, that zygotic ptk7^{hsc9/hsc9} embryos look morphologically normal, at least until 3 days post fertilization. Examination of CoPA pathfinding in zygotic ptk7^{hsc9/hsc9} embryos using 3A10 immunostaining revealed that 100% of CoPA axons turned anteriorly and 0% turned posteriorly (n=83; 18 embryos)(Figure 1). The Ciruna lab had reported that maternal transcripts of ptk7 are extremely long-lived and rescue defects in early zygotic embryos. We therefore examined CoPA pathfinding in maternal-zygotic (MZ) ptk7^{hsc9/hsc9} embryos. To accomplish this, we bred ptk7^{hsc9/hsc9} homozygous mutant females with ptk7^{hsc9/+} heterozygous males to generate MZptk7^{hsc9/hsc9} embryos. MZptk7^{hsc9/hsc9} embryos were noticeable because they exhibit a convergence extension phenotype, as first reported by the Ciruna Lab. We found that approximately 48% of the CoPAs turned correctly anteriorly, while 52% of the CoPAs turned incorrectly posteriorly in MZptk7^{hsc9/hsc9} embryos (n=110; 18 embryos) (Figure 2). Despite a defect in A-P guidance of CoPA cells, all CopA axons still projected ventrally, crossed the midline, and extended back dorsally to
join the DLF. This suggests that loss of ptk7 does not affect dorsal-ventral
guidance, but has a specific role in controlling the A-P guidance of CoPA cells. We
concluded that Ptk7 is required for the proper anterior turning of CoPA fibers.

**Celsr3 is required for proper CoPA axon pathfinding**

Previous studies in mice demonstrated that pathfinding of commissural axons is
randomized in Celsr3 knockout mice (Shafer et al., 2011). We therefore tested the
role of Celsr3 in anterior guidance of CoPA fibers in zebrafish. To accomplish this,
we made use of *Tg (tbx16: GFP)* fish that express GFP in CoPA neurons and
allows the visualization of CoPA pathfinding using live imaging of embryos at 48
hpf. It should be noted that tbx16 is a transcription factor expressed in mesoderm,
but due to position effects of transgene insertion, GFP is expressed in CoPA cells.
To ensure that the GFP-positive cells in *Tg (tbx16: GFP)* fish were indeed CoPA
cells, we first examined morphology of these neurons. GFP-expressing neurons
had T-shaped morphology with a single axon that crossed the midline and
projected anteriorly. They also displayed two characteristic dendrites, one that
projects anteriorly and one that projects posteriorly. Next, we co-stained the
transgenic fish with 3A10 antibody, our marker for CoPA cells at 30 hpf. We found
that all GFP-expressing neurons in the spinal cord at 30hpf were also 3A10-
positive, suggesting that these cells are indeed CoPA neurons. Interestingly, not
all 3A10-positive CoPA cells were GFP-positive, suggesting that the transgene
insertion is not capable of driving expression in all CoPA neurons (Figure 3).
We next tested the role for celsr3 in CoPA pathfinding using Tg(tbx16:GFP) fish. To do this, a celsr3 MO was injected into 1-cell stage embryos of Tg(tbx16:GFP) transgenic fish and they were mounted laterally and their CoPAs were analyzed for pathfinding defects using live imaging. This morpholino has been previously used to phenocopy celsr3 mutants. We found that approximately half of the CoPAs turned incorrectly posteriorly, while the other half turned correctly anteriorly (n=102; 12 embryos) (Figure 4). As a control, CoPAs were analyzed in uninjected Tg (tbx16: GFP) fish and 100% of the CoPAs turned correctly anteriorly (n=79; 10 embryos).

**JNK is required for proper CoPA axon pathfinding**

As described above, JNK is thought to be an effector of PCP in several cell contexts. We therefore tested the role of JNK in anterior guidance of CoPA fibers. We treated 15 hpf embryos with 1um concentration of SP600125, a pan JNK inhibitor, until the embryos reached 24 hpf, after which the inhibitor was washed off and the embryos were allowed to survive until 48 hpf. After analyzing 171 CoPAs in 19 embryos we found that approximately 53% of the CoPAs turned correctly anteriorly, while 47% of the CoPAs turned incorrectly posteriorly (n=171; 19 embryos) (Figure 5). Similar to ptk7 mutants and celsr3 morphants, we did not observe any defects in midline crossing or D-V pathfinding decisions. These results confirm that JNK is a necessary effector of PCP in axon guidance decisions.
**Glypican 4 is not required for proper CoPA axon pathfinding**

To test the role for Glypican4, we examined CoPA pathfinding in *knypek/gpc4*<sup>fr6</sup> mutants. These mutants exhibit a convergence extension defect in which the embryos develop shorter and wider due to a defect in PCP-mediated cell movements. However, we found that 100% of the CoPAs (n=80; 10 embryos) turned anteriorly correctly in *knypek/gpc4*<sup>fr6</sup> fish as seen in WT embryos (Figure 6). We concluded that Glypican4 is not required for proper anterior turning in CoPA neurons.

**Disrupting Wnt5b gradient does not affect CoPA axon pathfinding**

A gradient of Wnt5b has been proposed to exist in a high to low gradient A-P. However, when we disrupted this gradient by heatshocking Tg(*hsp70:Wnt5b:GFP*)<sup>w33</sup> embryos which causes overexpression of Wnt5b(pipetail) ubiquitously in the embryo, we observed no CoPA pathfinding defects (n=70) One-hundred percent of the CoPAs turned correctly anteriorly like wildtypes (Figure 7) (Hammerschmidt et al., 1996).

**Midline crossing is not required for subsequent pathfinding decisions in CoPA neurons**

We have previously shown that the anterior pathfinding in pre-midline crossing fibers is influenced by Scribble, a PCP protein. We extended these findings to other PCP proteins by testing the role of Fzd3a in pre-midline anterior guidance. Our preliminary data demonstrated that CoPA axons lose A-P directionality in *fzd3a*<sup>rw689</sup> mutants, with approximately half of neurons pathfinding posteriorly...
incorrectly. We then tested whether anterior pathfinding of ipsilateral CoPAs was dependent on PCP signaling. First, injection of DCC MOs into WT embryos prevented some CoPA axons from crossing the midline. These affected CoPA axons, however, still extended anteriorly in the ipsilateral spinal cord, suggesting that midline crossing is not required for CoPA axons to become responsive to A-P guidance cues. Upon closer examination of dcc-morphant embryos, we noticed that a small proportion of CoPA cells inappropriately turned posteriorly (~10%) of axons growing posteriorly. After analyzing \textit{fzd3a}^\textit{rw689/rw689} homozygous mutant embryos post DCC MO injection, we found that approximately 56% of the CoPAs turned correctly anteriorly, while 44% turned incorrectly posteriorly (n=109; 18 embryos) (Figure 8). Taken together, our results demonstrate two conclusions: i) that midline crossing is not required for subsequent A-P guidance decisions, ii) the anterior guidance of pre-crossing CoPA axons is dependent on PCP components.

**Discussion**

Taken together, data from this thesis and previous work from the Walsh lab have now identified four transmembrane PCP proteins Fzd3a, Vangl2, Ceslr3, and Ptk7, as well as 2 downstream effectors of PCP: Scribble and JNK, as essential for the anterior-posterior guidance of CoPA axons. With single neuron resolution, we showed that loss of \textit{fzd3a} or \textit{vangl2} or \textit{scribble} or \textit{Celsr3} or \textit{JNK} or \textit{Ptk7} leads to randomized growth of CoPA axons along the A-P axis (Figure 8). Interestingly, CoPA axons do not wander or stall, but grow and extend for long distance in either
the anterior or posterior direction. Together, these data support two major conclusions, i) PCP proteins are not required for axon growth per se, but are essential for proper anterior turning of commissural axons, and ii) PCP proteins do not affect dorsal-ventral guidance decisions or midline crossing by commissural axons. These findings strongly support a role for PCP in interpreting anterior-posterior guidance cues. How does PCP control the steering of CoPA axons? One possibility is that Wnt, expressed in a gradient, attracts CoPA axons like a classic secreted chemoattractant. Interestingly, we observed no knotting or stalling of CoPA axons in any of the PCP mutants or morphants, which may argue against a simple model of Wnt acting as a chemoattractant attracting the axons anteriorly. One would expect the CoPAs to knot or stall if the chemoattractive gradient guiding them is disrupted. Our findings do not entirely rule out a role for Wnt ligands as secreted chemoattractants in guiding commissural axons; however, we find no role for the Wnt co-receptor, Glypican 4 in mediating PCP functions in CoPA axon pathfinding, and we saw no observable axon pathfinding defects when we disrupted the Wnt5b gradient in vivo. This is interesting, since Glypican 4 has been reported as essential for other PCP processes in zebrafish including convergence extension movements of the mesoderm and neuroectoderm. Also, a Wnt5 gradient has been proposed to exist in a high-low A-P fashion. However, we do find that Fzd3a and Ptk7 are required, which may be sufficient to mediate all of Wnt actions in growth cone steering. An alternative hypothesis is that Wnts
function as permissive cues. They need to be present to engage the PCP machinery, but may not function as chemoattractants. To date, no knockout studies have implicated Wnts in commissural pathfinding likely due to the large number of Wnts (19) that exist.

If Wnts are not instructive in guidance along the anterior-posterior axis, what signal is responsible for telling CoPA axons to grow? It is clear that in the absence of PCP they extend for long distances, some in the right direction, others in the wrong direction. It seems likely that there must be other extrinsic guidance cues that control CoPA axon growth along the A-P axis. Otherwise, we would predict that CoPA axons would never leave the segment where the cell body is located. They would simply cross the midline and stop pathfinding in the contralateral cord without ever turning anteriorly or posteriorly. Therefore, is PCP better thought of as a signaling pathway that actively instructs growth cone steering through its regulation of the actin cytoskeleton or is PCP better thought of as a geometric scaffold or compass inside the growth cone that allows growth cones to respond appropriately to other guidance cues in the extracellular environment? It is possible that we need further genetic screens to uncover additional cues that control CoPA axon growth and pathfinding.

An additional caveat that that should be mentioned is it remains possible that PCP components are required in the environment to control axon pathfinding. It is well established that neuroepithelial cell and midline floorplate cells are planar
polarized along the anterior-posterior axis. It remains possible that neuroepithelial cells need to be polarized to properly secrete or present molecules on the surface that growing CoPA axons require for pathfinding. Future experiments will be aimed at determining which cells, CoPA neurons or the cells in the surrounding environment require PCP signaling components for proper axon pathfinding. We propose three models of PCP-mediated axon guidance. The first model requires PCP in the growth cone of the axon and requires a Wnt gradient in the environment in order for the axon to make the correct anterior turn (Figure 10A). However, this model may not be correct considering overexpressing Wnt5b did nothing to CoPA axon pathfinding. Perhaps there is a gradient of Fzd receptors instead of the Wnt ligand itself. Alternatively, there could be a gradient of Secreted Frizzled Related Proteins (SFRPS) that bind to Wnts that is responsible for this anterior turning. Further experiments need to be done in order to confirm this. The second model requires PCP within the growth cone in order for the growth cone to make the proper turning decisions, with a different ligand responsible for axon extension (Figure 10B). The third model requires PCP both in the growth cone of the axon and in the environment (neuroepithelial cells), which allows the growth cone to constantly realign itself with its polarized environment (Figure 10C). In summary, transplant experiments need to be performed in order to determine a cell-autonomous role or non cell-autonomous role of PCP in axon guidance to determine which model is correct.
Future directions

Previous transplant studies have been performed on facial branchiomotor neurons in the hindbrain of zebrafish to determine a cell-autonomous or non-cell-autonomous role of PCP, as a means to determine if PCP is required within the neuron or within the environment (Walsh et al., 2011). These two models are not mutually exclusive, as they both can be required concurrently. In fact, for neuron migration, PCP gene function is now known to be required in both within the neuron and the environment (Walsh et al., 2011). In order to determine if PCP is required cell-autonomously or non-cell autonomously in commissural neurons, we plan to perform cell transplant experiments in which we transplant wildtype CoPAs into a PCP mutant environment and vice-versa. This will allow us to determine whether PCP is required within the neuron, within the environment, or both.

Additionally, we plan to determine the mechanism of PCP and its downstream effector’s actions by inhibiting JNK in Fzd3a mutants and seeing if activated JNK can rescue defects in Fzd3a mutants. Also, since addition if JNK inhibitors would block JNK function in both neurons and the environment, we plan to express dominant negative JNK specifically within CoPA neurons to determine whether JNK functions cell-autonomously. Although we know Ptk7 is required for proper anterior turning in CoPA neurons, we still do not know what domain is required for
its function in pathfinding. This is relevant since some studies support a role for the cytoplasmic domain in activating Src family kinases, whereas other studies argue that the cytoplasmic domain is dispensable. In order to determine this, we will perform rescue experiments in which we inject the extracellular domain alone, extracellular domain with the Ptk7 transmembrane domain substituted with the transmembrane domain of EGFR, and the intracellular pseudokinase domain of Ptk7 into one-cell stage Ptk7 mutant embryos in order to determine if Ptk7 mutants can be rescued by these constructs respectively. This will allow us to determine which domain of Ptk7 is required for its function in COPA axon pathfinding.
Figure 1: Distribution of Planar Cell Polarity Proteins
Distribution of core and accessory planar cell polarity proteins including transmembrane and cytosolic proteins, as well as downstream effectors. The core PCP proteins include: Frizzled (Fzd), Celsr, Van Gogh-like (Vangl), Disheveled (Dvl), and Prickle (Pk). The Wnt co-receptors include Glypican 4 (Gpc4) and Protein Tyrosine Kinase 7 (PtK7). The downstream effectors include Rho, Rac, and Jun N Terminal Kinase (JNK).
Figure 2: *Ptk7* is required for CoPA axon pathfinding. At 33 hpf, CoPA axon pathfinding is complete. (A-C)Lateral and dorsal confocal maximum intensity projections of 3a10 immunofluorescence in the spinal cord of (A) wild-type, (B) MZ*ptk7*^[hsc9/hsc9]^. Red arrows indicate CoPAs pathfinding incorrectly posteriorly. Asterisks indicate CoPAs pathfinding correctly anteriorly. In all images, anterior is to the left and posterior is to the right unless otherwise noted. (C) Percentage comparisons of CoPA axon projections after crossing the midline of all mentioned between WT and MZ*ptk7*^[hsc9]^- genotypes (n=number of CoPA neurons).
Figure 3: Live-imaging versus immunostaining CoPAs
Tbx16: GFP transgenic fish express GFP in CoPA neurons. This provides a means to live-image embryos using spinning disk confocal microscopy to screen for mutants (A) Lateral and orthogonal projections of *tbx16: GFP* at 40 hpf
(B) Merged images of 3a10 antibody staining and GFP expression in *tbx16: GFP* transgenic fish
Figure 4: Celsr3 is required for CoPA axon pathfinding. Lateral confocal projects of GFP-expressing CoPA neurons in the spinal cord of tbx16:GFP fish at 42 hpf of A) wild-type and (B) Celsr 3 MO injected at 18 ng concentration. Asterisks denote CoPA neurons turning incorrectly posteriorly (C) Quantification of the percentage of neurons that turned either anteriorly or posteriorly (n = number of CoPA neurons)
Figure 5: JNK is required for CoPA axon pathfinding. Lateral confocal projects of GFP-expressing CoPA neurons in the spinal cord of tbx16: GFP fish at 42 hpf of A) wild-type and (B) JNK inhibitor (SP600125) treated at 1uM concentration. Asterisks denote CoPA neurons turning incorrectly posteriorly (C) Quantification of the percentage of neurons that turned either anteriorly or posteriorly (n = number of CoPA neurons)
Figure 6: Glypican 4 is not required for proper anterior pathfinding in CoPAs. At 33 hpf, CoPA axon pathfinding is complete. (A-B) Lateral confocal maximum intensity projections of 3a10 immunofluorescence in the spinal cord of (A) wildtype and (B) knypek<sup>fr6/fr6</sup> mutants. 100% of CoPAs in knypek<sup>fr6/fr6</sup> mutants turned anteriorly like wildtypes. n=72. ( n =number of CoPA neurons).
Figure 7: Overexpressing Wnt5b does not affect CoPA axon pathfinding At 33 hpf, CoPA axon pathfinding is complete. (A-B) Lateral confocal maximum intensity projections of 3a10 immunofluorescence in the spinal cord of (A) wildtype and (B) Tg(hsp70:Wnt5b:GFP)w33. 100% of CoPAs in Tg(hsp70:Wnt5b:GFP)w33 turned anteriorly like wildtypes. n=70 (n=number of CoPA neurons).
Figure 8: Midline crossing is not required for subsequent CoPA axon pathfinding decisions. At 33 hpf, CoPA axon pathfinding is complete. (A-C) Lateral and dorsal confocal maximum intensity projections of 3a10 immunofluorescence in the spinal cord of (A) wild-type+DCC MO, (B) fzd3a<sup>nw689/nw689</sup>+DCC MO, and (C) scrib<sup>nw468/nw468</sup>+DCC MO. All DCC MO were injected at 3ng concentration. Red arrows indicate CoPAs pathfinding incorrectly posteriorly. Asterisks indicate CoPAs pathfinding correctly anteriorly. Dotted line indicates midline of embryo. In all images, anterior is to the left and posterior is to the right unless otherwise noted. (D) Percentage comparisons of CoPA axon projections after crossing the midline of all mentioned genotypes (n=number of CoPA neurons).
Figure 9: PCP components are required for proper CoPA axon pathfinding. Comparison of PCP post-crossing phenotypes. Wildtype and zygotic ptk7^{hsc9/hsc9} mutants exhibit 100% of CoPA neurons turning anteriorly, with pk1b^{fh122/fh122} serving as the control, as pk1b^{fh122/fh122} is not expressed in the spinal cord, while other PCP mutants exhibit approximately 50% of CoPA neurons turning incorrectly posteriorly (n=number of CoPA neurons).
Figure 10: Possible models of PCP guidance of CoPAs
Comparison of possible PCP-mediated axon guidance mechanisms. (A) The first model requires PCP in the growth cone being guided by a Wnt gradient for anterior growth cone turning. (B) The second model requires PCP in the growth cone for steering but requires another cue for axon extension. (C) The third model requires PCP in the growth cone and in the surrounding environment (neuroepithelial cell) for growth cone steering but requires another cue for axon extension.
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