Investigating the Relationship Between Cilia and Planar Cell Polarity Signalling During Zebrafish Development

by

Antonija Borovina

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Graduate Department of Molecular Genetics
University of Toronto

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2013

Abstract

Cilia are microtubule-based organelles that project into the extracellular space and have various functions including transducing sensory information, regulating developmental signalling pathways, and generating directed fluid flow, making them important regulators of vertebrate development and homeostasis [1]. Despite their importance, there are many aspects of cilia formation and function that remain poorly understood. The planar cell polarity (PCP) pathway is a branch of Wnt signalling that provides positional information to cells and is required for polarized morphogenic cell movements. Previous studies of PCP effector proteins suggested that PCP signalling was required for cilia formation. However, these proteins are not specific to the PCP pathway and are shared with other branches of Wnt signalling [2, 3]. To determine the role of a core and specific PCP regulator on ciliogenesis, I examined maternal-zygotic (MZ) vangl2 zebrafish mutants using an in vivo marker of cilia, Arl13b-GFP. Analysis of MZvangl2 mutants revealed that PCP is not required for cilia formation but is required for the posterior tilting and posterior positioning of motile cilia, essential for directed fluid flow. A parallel branch of studies suggested that cilia are actually required to regulate PCP signalling because defects in PCP-mediated morphogenic movements were observed with the knockdown of certain proteins that localize at or near cilia or basal bodies [4-8]. To determine whether cilia were required to
establish PCP, I generated MZ-intraflagellar transport-88 (IFT88) mutants, where ciliogenesis is completely abolished. Analysis of MZift88 mutants revealed that cilia are not directly required for PCP-mediated morphogenic movements. However, I observed that MZift88 mutants had defects in oriented cell divisions (OCD) occurring during gastrulation. Remarkably, these divisions occur prior to cilia formation, suggesting a cilia-independent role for IFT proteins in cell divisions, which may have important consequences on the interpretation of the role of cilia in disease.
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To my parents Ina and Robert, I cannot describe how thankful I am for all your unconditional love and support. Every decision you have made has been for my best interest and any success I have is as much yours as it is mine. To my brother Andro, thank you for being a continuous source of love and laughter, and to my grandparents in Croatia who have always been so proud of me. Finally, to Max, I am so grateful to have you in my life and thank you for always believing in me.
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<td>AB</td>
<td>Apical-Basal</td>
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<td>ADPKD</td>
<td>Autosomal Dominant Polycystic Kidney Disease</td>
</tr>
<tr>
<td>AP</td>
<td>Anterior-Posterior</td>
</tr>
<tr>
<td>ARPKD</td>
<td>Autosomal Recessive Polycystic Kidney Disease</td>
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<tr>
<td>AV</td>
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<tr>
<td>BBS</td>
<td>Bardet Biedl Syndrome</td>
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<td>Ca⁺²</td>
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<td>Cluster of Differentiation 3</td>
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<td>CE</td>
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<td>ENU</td>
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<td>Erk</td>
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F-actin, Filamentous Actin
Fmi, Flamingo
Fz, Frizzled
Gai, Alpha Subunit of Adenylyl Cyclase Inhibitory Heterotrimeric G Protein
GFP, Green Fluorescent Protein
Gli, Glioma
GLR, Germline Replacement
GRP, Gastrocoel Roofplate
GTP, Guanosine Triphosphate
Hh, Hedgehog
IFT, Intraflagellar Transport
IFT88, Intraflagellar Transport Protein 88
IHC, Immunohistochemistry
Kif3a, Kinesin Family Member A
KV, Kupffer’s Vesicle
LGN, Leucine Glycine Asparagine Containing Protein
Lrrc6l, Leucine Rich Repeat Containing 6-Like
MEF, Mouse Embryonic Fibroblast
Mek, MAPK/ERK Kinase
MO, Morpholino
ML, Medio-Lateral
MZ, Maternal Zygotic
NMD, Nonsense-Mediated Decay
NRH1, Neutrophin Receptor Homolog 1
NuMA, Nuclear Mitotic Apparatus Protein

OCD, Oriented Cell Division

Ofd1, Orofaciodigital Syndrome 1

ORPK, Oak Ridge Polycystic Kidney

Plk1, Polo-Like Kinase 1

Ptc, Patched

PCD, Primary Ciliary Dyskinesia

PCP, Planar Cell Polarity

PCR, Polymerase Chain Reaction

PDGF, Platelet Derived Growth Factor

PDGFR, Platelet Derived Growth Factor Receptor

PDZ, Post Synaptic Density Protein (PSD95), Drosophila Disc Large Tumor Suppressor (Dlg1), and Zonula Occludens-1 Protein (Zo-1)

Pk, Prickle

PKD, Polycystic Kidney Disease

PKD1, Polycystin 1

PKD1L1, Polycystic Kidney Disease 1 Like 1

PKD2, Polycystin 2

Ptk7, Protein Tyrosine Kinase 7

RanGTP, Ras Related Nuclear Protein-Guanosine Triphosphate

RhoA, Ras Homolog gene Family Member A

Ror2, Receptor Tyrosine Kinase-Like Orphan Receptor 2

RT, Reverse-Transcriptase

Ryk, Related To Receptor Tyrosine Kinase
Sec8, Secretory Protein 8
Smo, Smoothened
Stbm, Strabismus
Sufu, Suppressor Of Fused
TCR, T-Cell receptor
TEM, Transmission Electron Microscopy
Vangl, Van Gogh Like
Waif1, Wnt-Activated Inhibitory Factor 1
Wnt, Wingless-Type MMTV Integration Site Family
WT, Wild-Type
Xdd1, *Xenopus* Dishevelled Deletion 1
YFP, Yellow Fluorescent Protein
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Chapter 1

1. Introduction

1.1 Overview of Cilia and PCP Signalling

The cilium is a unique organelle that is present on virtually every vertebrate cell. Despite the fact that their existence has been known for roughly 300 years, it is only in the last decade or so that cilia have become the focus of a flood of new research [9]. Traditionally cilia were believed to have few functions, mainly in generating fluid flow, and in sensory roles on specialized cells. Studies in model organisms have been the driving force for uncovering new functions of cilia including implicating them in developmental signalling pathways like Hedgehog (Hh), Platelet Derived Growth Factor (PDGF) and Wnt. Importantly, these studies have associated cilia dysfunction as an underlying cause of human disease, one of the first being polycystic kidney disease (PKD). Today it is believed that as many as 100 rare human disorders may in some part have a ciliary basis [1]. These disorders are termed ciliopathies and have a collective incidence as high as 1:1000 births [10]. In light of these findings, it is hard to believe that cilia were once considered vestigial organelles that were merely evolutionary remnants.

Despite their obvious importance, the mechanisms responsible for cilia formation are not fully understood, but recent studies point to planar cell polarity (PCP) signalling as a key factor [11]. PCP signalling is an evolutionarily conserved signalling pathway that was discovered in Drosophila and was found to provide positional information to cells. PCP signalling has emerged as an important determinant of vertebrate embryogenesis in its own right, since PCP dysfunction can lead to deafness, neural tube closure defects, heart defects, and PKD [12]. As the relationship between PCP signalling and ciliogenesis began to emerge, it was complicated by studies that suggested that cilia were required for PCP signal transduction [11]. Both sides of the argument had compelling evidence and elucidating the relationship between cilia and PCP signalling became a heavily studied topic in developmental biology. Here I address the inter-connections between the two, and describe the advances that have been made at providing a unifying model of how cilia and PCP are related.
1.1.1 PCP signalling in Drosophila

PCP is a type of polarity that is most easily described in a sheet of epithelial cells. It is well known that epithelial cells possess apical-basal (AB) polarity, distinct domains that differ in protein composition. PCP occurs orthogonal to AB polarity, or in the plane of a sheet of cells. Most of what is known about how PCP is established comes from studies in Drosophila. In flies, the wing, eye, and abdomen all show obvious planar polarity in the uniform arrangement of their bristles and hairs, and have been useful read-outs of PCP defects [13].

PCP is established by core PCP factors that include the transmembrane proteins Strabismus (Stbm; Vangl in vertebrates), Frizzled (Fz), and the atypical cadherin Flamingo (Fmi; Celsr in vertebrates), as well as the cytoplasmic proteins Dishevelled (Dvl), Diego (Dgo) and Prickle (Pk). Core PCP factors have been shown to asymmetrically localize in polarized tissues. In the case of the wing epithelium, each cell has a single actin-rich hair emanating from the distal end of its apical surface. Correspondingly, Stbm and Pk were found to accumulate at the proximal end of the apical surface, while Dvl, Dgo, and Fz were found at the distal end [14]. Fmi is localized all along the apical membrane and Fmi homodimers are required to promote asymmetries on adjacent cells [15]. At the distal end, Dvl is recruited to the membrane by Fz, which is required for its function in PCP signalling. Membrane associated Dvl ultimately leads to cytoskeletal reorganization that is required for hair growth initiation at the distal end [16]. Stbm and Pk restrict Fz/Dvl-mediated cytoskeletal rearrangements to one side of the cell by competing for Dvl and affecting its membrane localization (Figure 1.1.1), [17, 18].

In order for uniform planar polarity to be established across the tissue, the polarity within an individual cell must be coordinated with neighbouring cells. PCP signalling possesses a property called domineering non-autonomy that reinforces intercellular polarity through the interactions of PCP components on opposite cells [19]. This was demonstrated through genetic mosaics where Stbm or Fz mutant cell clones were studied in relation to WT cells of the abdominal epidermis. These mutant clones were shown to disrupt the polarity of bristles on surrounding WT cells, however not always in the same manner. For example, Fz loss-of-function mutant clones reorient the outgrowth of bristles toward the mutant clone, but Stbm loss-of-function mutant clones reorient bristles away from the clone [20]. An upstream morphogen gradient is believed to initially establish polarity and cells align their polarity by detecting differences in the morphogen
levels of their neighbours. Since Fz proteins are common receptors for Wnt ligands, it was proposed that the morphogen was a Wnt, but in *Drosophila* that does not appear to be the case and the unidentified morphogen is termed *Factor X* [21].

It should be noted that the core PCP factors are not the only proteins required to establish polarity. Downstream of the core factors are proteins termed *PCP effectors*, the most notable being Inturned, Fuzzy, and Fritz. These factors are tissue specific and are required to interpret and execute the polarity established by the core factors [22]. When discussing PCP in flies, one must also consider the Fat-Dachsous (Ds) pathway. Fat is a very large atypical cadherin that controls PCP by binding to Ds, and disruption of either of these proteins leads to both autonomous and non-autonomous polarity defects. Analysis of specific polarized tissues showed that Ds is expressed in gradients that could provide spatial cues, but it is still unclear how these factors fit in with core PCP factors. Early studies suggested that Fat-Ds were upstream of core PCP signalling but more recent studies suggest that they may be parallel pathways. However, the precise relationship between Fat-Ds and core PCP signalling factors in establishing PCP still needs to be determined (reviewed in Sopko and McNeill, 2009) [23].
Figure 1.1.1 PCP Signalling in the *Drosophila* wing epithelium. PCP occurs orthogonal to AB polarity and is established by the asymmetric distribution of core PCP factors. Wing epithelial cells show distinct planar polarity in the proximal-distal axis, where a single actin-rich hair emanates from the distal end of the apical surface. Dishevelled, Diego, and Frizzled accumulate at the distal side where they induce an enrichment of apical actin that is required for hair growth initiation. Conversely, Strabismus and Prickle accumulate at the proximal side and are required to restrict Dishevelled-mediated cytoskeletal rearrangements to the distal side. Flamingo is localized all along the apical membrane and forms homodimers that are required to promote asymmetries on adjacent cells.
1.1.2 Core PCP factors in vertebrates

In vertebrates, the number of core PCP homologs has been expanded so elucidating the function of these factors has been more challenging than in Drosophila [24]. Nonetheless, vertebrate studies have revealed new and important functions of PCP signalling in very diverse tissues. To date, PCP signalling is has been implicated in controlling oriented cell movements and divisions required during gastrulation, neurulation, and eyelid closure; polarizing epithelial cells in the node, inner ear and epidermis; controlling the growth and guidance of axons and dendrites; and in controlling the architecture of tubular organs like the kidney [25].

The first evidence of a role for core PCP factors in vertebrate development came from studies in Xenopus and zebrafish [24]. Vertebrate homologs of Drosophila PCP proteins were shown to regulate convergence and extension (CE) movements occurring during gastrulation and neurulation [8, 26-33]. CE movements are a combination of cell behaviors that ultimately lead to the extension of the vertebrate body axis and enable proper neurulation. During gastrulation, PCP controls medio-lateral cell intercalations, directed cell migration, and radial intercalations that narrow the developing embryo in the medio-lateral axis and lengthen it in the anterior-posterior axis (Figure 1.1.2a-c), [27, 34, 35]. Analysis of the zebrafish trilobite mutant showed that Vangl2 functions both cell-autonomously and non-autonomously to regulate CE movements [27]. This indicated that domineering nonautonomy is characteristic of vertebrate PCP signalling as well. Additionally, PCP signalling has been shown to regulate oriented cell divisions (OCD) occurring in dorsal tissues of zebrafish embryos undergoing gastrulation. PCP signalling is required to properly orient these divisions so they occur in the animal-vegetal direction, further contributing to axis elongation [36].

The first, and most well characterized PCP mutant in the mouse is the loop-tail/vangl2 mutant. The most obvious defect in these mutants is an open neural tube in the hindbrain and spinal region, termed craniorachischisis [37, 38]. This particular type of neural tube closure defect also stems from disrupted CE movements, and has subsequently been established to be a hallmark of disrupted PCP signalling in mice and Xenopus [39]. In order for a hollow neural tube to form, the flat neural tube precursor, the neural plate, must bend, and the resultant folds must fuse. CE movements are required to narrow the distance between the folds, thus allowing them to fuse (Figure 1.1.3a), [40]. The importance of the above findings was emphasized by two recent
reports that found mutations in \textit{vangl1} and \textit{vangl2} in human patients exhibiting neural tube closure defects [41, 42].

PCP signalling also plays a role during zebrafish neurulation but the situation differs because zebrafish neurulation proceeds using an alternative mechanism. Here neural tube formation also begins with a flat neural plate that starts to fold toward the midline. This creates a transient structure called the neural keel, where the apical surfaces of cells from opposite sides of the neural plate are facing each other. At this stage, cells are undergoing medio-lateral intercalations and cell divisions across the neural keel midline. Eventually, cells on opposing sides of the midline begin to retract their apical surfaces to establish a lumenized neural tube (Figure 1.1.3b), [43]. PCP signalling has been shown to reestablish polarity that is transiently lost during cell division and is required for daughter cells to reintegrate into the contralateral side of the neural keel. Analysis of maternal-zygotic (MZ) \textit{vangl2} mutants revealed disrupted intercalation of daughter cells that results in ectopic accumulations of neural progenitors at the midline [43].
Figure 1.1.2 CE movements occurring during gastrulation lead to the lengthening and narrowing of the vertebrate body axis. (a-c) Individual cell behaviors differ depending on the region of the embryo and include medio-lateral intercalations (a), radial intercalations (b), and directed cell migrations (c).
Figure 1.1.3 Neural tube formation in the mouse and zebrafish. The precursor to the neural tube is a flat structure called the neural plate. (a) During mouse neurulation, the neural plate bends at specific hinge points to form neural folds, which subsequently fuse to form a hollow neural tube. CE movements are required to narrow the distance between the folds, enabling them to fuse properly. (b) During zebrafish neurulation, the neural plate folds toward the midline to form a transient structure called the neural keel. In the neural keel, the apical surfaces of cells of opposite sides of the neural plate face each other and undergo medio-lateral cell intercalations and cell divisions. PCP signalling is required to reestablish polarity after cell division and for daughter cells to reintegrate on the contralateral side of the neural keel. The neural keel eventually forms a narrow structure called the neural rod, and at this point, the apical surfaces of cells on apposing sides of the neural midline retract to form a lumenized neural tube.
1.1.3 Other PCP regulators in vertebrates

In addition to the core PCP factors discovered in *Drosophila*, vertebrate studies have revealed additional regulators that promote PCP signalling. First and foremost are the secreted glycoproteins, Wnt5a and Wnt11, which were shown to regulate CE movements during gastrulation [29, 44-46]. This was interesting given the fact that Wnts were shown not to play a role in *Drosophila* PCP signalling [21]. The Wnt response in PCP signalling does not signal through the traditional (canonical) Wnt pathway, even though many of the components are shared including Wnt, Fz, and Dvl proteins [29, 44]. Canonical Wnt signalling involves Wnt ligands binding to Fz receptors, leading to the recruitment of Dvl and the stabilization of β-catenin. β-catenin subsequently translocates to the nucleus where it activates canonical Wnt target genes (Figure 1.1.4), [47]. PCP signalling is termed non-canonical Wnt signalling because it also involves Wnt, Fz, and Dvl proteins, but it does not initiate a transcriptional response. Instead, it leads to cytoskeletal rearrangements by activating small GTPases (Figure 1.1.4). Dvl is composed of three distinct domains, the DIX domain, the PDZ domain, and the DEP domain, that differ in the intracellular pathways they activate. The DIX and PDZ domains are required to activate canonical Wnt signalling by inducing the stabilization of cytoplasmic β-catenin [16]. The PDZ and DEP domains are required for PCP signalling since they regulate the translocation of Dvl to the membrane and the activation of Rac GTPase [16, 48]. In addition, the Wnt-Ca\(^{++}\) pathway is another branch of non-canonical Wnt signalling that also involves Wnt, Fz, and Dvl proteins, but leads to an increase in intracellular Ca\(^{++}\) and activates the NF-AT transcription factor (Figure 1.1.4), [49].

It is still unclear as to how the specificity of a particular Wnt pathway is determined since an individual Wnt ligand cannot be classified as either canonical or non-canonical. It now appears that this process is both context specific and involves additional regulators [47]. One such regulator is the heparin sulfate proteoglycan, Knypek/Glypican 4. Analysis of zebrafish *knypek* mutants showed that Knypek was required for CE movements and that it functionally interacts with, and potentiates, Wnt11 signalling [50]. In addition to Knypek, other factors including Ror2, PTK7, Cthrc1, Waif1, Ryk, and NRH1 have also been shown to selectively activate the PCP pathway [51-56].
Figure 1.1.4 Wnt signalling is divided into distinct branches though many of the components are shared. (a) Canonical Wnt (β-catenin) signalling involves Wnt ligands binding to Frizzled receptors, causing them to associate with the Lrp5/6 co-receptor. This interaction recruits Dishevelled, and sequesters members of the β-catenin destruction complex (APC-Axin-GSK3). This prevents the degradation of β-catenin, allowing it to accumulate and translocate to the nucleus where it activates the expression of target genes. (b) Wnt-Ca\(^{2+}\) signalling also involves Wnt ligands, Frizzled, and Dishevelled, but leads to an increase in intracellular Ca\(^{2+}\). Ca\(^{2+}\)-sensing proteins like CaMKII and PKC are required to interpret intracellular Ca\(^{2+}\) and activate the NF-AT transcription factor. (c) The PCP pathway involves Wnt ligands binding to Frizzled, an interaction that also involves additional regulators like Knypek, and leads to the recruitment of Dishevelled. Dishevelled then interacts with the scaffolding protein Daam1 and recruits downstream factors like RhoA, ultimately resulting in cytoskeletal remodeling. Changes in the cytoskeleton are restricted to one side of a cell by Vangl2 and Prickle because they compete for the binding of Dishevelled.
1.1.4 Asymmetric localization of PCP factors in vertebrates

Determining the localization of PCP factors has also proven to be more challenging in vertebrates than in *Drosophila*. Cells undergoing CE movements during gastrulation or neurulation have not yet established AB polarity, and the difficulty in visualizing the distribution of core PCP factors in these cells may be due to their dynamic nature [24]. This withstanding, some asymmetries have been observed. Explants of the dorsal marginal zone (DMZ) of *Xenopus* embryos were used to study cells undergoing CE movements during gastrulation. Expression of Venus-tagged *Xenopus* Dvl showed that it accumulated at the tips of elongated cells, where Dvl was suggested to be involved in localizing components necessary for actin polymerization and protrusive activity [57]. In zebrafish, GFP-tagged *Drosophila* Pk was observed in puncti along the anterior surface of cells undergoing neurulation but it was cytoplasmic in cells where polarity was disrupted [43]. The anterior distribution of GFP-*Drosophila* Pk was also seen in mesodermal cells undergoing CE movements, while GFP-tagged *Xenopus* Dvl was localized to the posterior side of these cells [35]. This observation that Pk and Dvl are localized to opposite sides of polarized cells is reminiscent of the localization pattern observed in the fly wing, suggesting a similar antagonistic relationship between these two factors [18].

A similar pattern was also observed in cells of the mouse node which have a single motile cilium emanating from their apical surface. These cilia are required to generate leftward fluid flow and initiate left-sided gene expression (see section 1.1.7). PCP signalling polarizes these cells so that each cilium grows from the posterior apical surface and is tilted in the posterior direction. In node cells, Pk2 and Vangl1 were shown to localize to anterior cell edges, while GFP-tagged Dvl2 and YFP-tagged Dvl3 were localized to the posterior side [58, 59].

The mammalian epidermis also shows a striking planar polarity with hair follicles aligned along the anterior-posterior axis. The *fz6* mouse mutant was shown to have a whorled hair pattern which is similar to the pattern of wing hairs in *Drosophila* Fz mutants [60]. When compared to E-cadherin, which uniformly labels cell borders, it was clear that Vangl2 and Celsr1 were enriched along the anterior-posterior axis [61].
Another example of a highly polarized tissue in vertebrates is the cochlea of the mammalian inner ear. Cochlear cells are epithelial cells that are arranged in rows, each containing a chevron-shaped stereociliary bundle. PCP signalling is required to coordinate the orientation of the stereociliary bundles across the tissue, and core PCP factors are asymmetrically distributed on the apical surface of cochlear cells. Initial observations reported that Fz3, Fz6, and Vangl2 were localized to the medial side, while Dvl2 was localized to the lateral side [62-64]. Fz3/6 being localized to the same side as Vangl2, and on the opposite side of Dvl2 was surprising, and suggested that cochlear cells establish intracellular polarity through a unique mechanism. However, mosaic labelling revealed that Fz6 was in fact localized to the lateral side, while Pk2 localized to the medial side [65]. These observations are in agreement with the pattern seen in the fly wing and underscore the importance of mosaic labelling when studying asymmetric localization patterns.

Some PCP factors including Vangl2, Dvl, and Inversin (Diego ortholog), have also been reported to localize to basal bodies and cilia [3, 7, 8]. This localization is consistent with some of the connections between cilia and PCP that are described below.

1.1.5 Cilia structure

The cilium is a long, thin microtubule-derived structure that is often referred to as the cell’s antenna because it integrates various extracellular signals and converts them into intracellular transduction cascades. Typically cilia are classified as either primary cilia or motile cilia, however these distinctions can be misleading. Primary cilia are always solitary and are usually immotile, but there are examples of motile primary cilia like those present in the mouse node. Motile cilia are those present on specialized multi-ciliated cells that often contain hundreds of beating cilia [66].

Structurally, the cilium consists of a shaft that is referred to as the axoneme which emanates from the centriole-derived basal body. The axoneme is the most defining feature of the cilium and a cross-section through it reveals a microtubule array arranged in a distinct cartwheel formation (Figure 1.1.5a). The outer portion of the axoneme is composed of nine parallel microtubule doublets that are arranged in a circle, and each doublet contains one complete tubule (A tubule)
and an incomplete one (B tubule), [67]. The doublets are connected to each other by nexin links and are extensively post-translationally modified with acetylations, glutamylations, and glycylations [68]. At the center of the axoneme is a central pair of microtubule singlets which are connected to the outer doublets by radial spokes, and collectively this structure is considered to have the 9+2 arrangement [69]. The central pair is believed to be involved in generating motility and is absent in most primary cilia, designating their structure as 9+0. There are exceptions here too, as some cilia exhibit motility despite having a 9+0 arrangement (http://www.primary-cilium.co.uk). Each microtubule doublet has inner and outer dynein arms that make contact with the adjacent doublet and cause the doublets to slide past each other (Figure 1.1.5a). Ciliary beating is generated by the sliding motion generated by dyneins, coupled to the mechanical constraints due to radial spokes and nexin links [70]. The axoneme is surrounded by the ciliary membrane which is continuous with the plasma membrane, however its composition is quite distinct. The cilium achieves its unique composition because it is in fact compartmentalized and there is a selective barrier for the entrance of proteins into the cilium [71]. The transition zone is a region that forms early during ciliogenesis and is characterized by the presence of Y-shaped linkers that tether the outer microtubule doublets to the ciliary membrane [72]. At the transition zone, the axoneme is in tight association with the ciliary membrane, allowing it to act as a gatekeeper for the cilium. Transition fibers are electron dense fibers that restrict diffusion between the cilium and the cytoplasm and are docking sites for proteins involved in cilia formation [73, 74]. Just above the transition zone is the ciliary necklace, a region identified by electron microscopy as having multiple strands of intramembrane particles which has also been suggested to play a role in limiting diffusion [75]. The root of the cilium sometimes appears to reside in an invagination of the plasma membrane termed the ciliary pocket. This pocket is not a characteristic of all cilia and is more often associated with immotile primary cilia. The function of the ciliary pocket remains unclear however it has been identified as a region of high vesicle trafficking, suggesting that it may be a docking site for vesicles destined for the cilium [76]. It has also been shown to be a region where the cilium interfaces with the actin cytoskeleton and this may have important consequences for cilia formation and function (reviewed in Satir and Christensen 2007), [77]. The ciliary basal body is also associated with distinct accessory structures called the basal foot and striated rootlet which emanate from the basal body and project into the cytoplasm. The precise function of these structures is still unclear, but they are
believed to link the cilium to the underlying cytoskeleton and perhaps stabilize it (refer to Chapter 4, Figure 1.1.5b), [78].

While the structure of the cilium may seem complicated, a review by Singla and Reiter (2006) effectively summarizes why cilia are in fact ideally suited for their purpose. They make three points: the fact that cilia project into the extracellular space provides them access to various environmental signals, their elongated shape provides a high surface to volume ratio that promotes the interaction of transmembrane receptors, and that the regulated entry of proteins into the cilia makes them specialized [79].

1.1.6 Cilia formation and maintenance

In most cells, ciliogenesis begins when the distal end of the mother centriole fuses to a golgi-derived ciliary vesicle (CV) and begins to migrate to the cell surface. While attached to the CV, the ciliary axoneme begins to elongate from the basal body, initiating formation of the ciliary membrane. At this point, the basal body also acquires transition zone-like features. At the cell surface, an enrichment of actin and certain transition zone proteins are required for proper docking of the basal body to the plasma membrane [72].

While hundreds of different cilia proteins have been identified, no protein synthesis occurs within the cilium itself. Since entry into the cilium is tightly regulated, proteins synthesized within the cytoplasm must be correctly targeted and transported into cilia. Also, the cilium is a highly dynamic structure and the balance of cilia assembly and disassembly establishes a steady state [80]. Furthermore, axonemal growth always occurs at the distal tip of the microtubules so a transport system is required to move cilia cargo, including axonemal components, along the length of the growing cilium. The process of intraflagellar transport (IFT) enables the bidirectional movement of ciliary cargo and is required for cilia assembly and maintenance [81]. This process is highly evolutionarily conserved and much of what we know about IFT can be attributed to studies in *Chlamydomonas* where disrupting IFT led to absent, shorter, or defective cilia [82]. IFT requires a multi-subunit adaptor complex called the IFT complex that binds ciliary cargo and associates with motor proteins to translocate along the cilium in the anterograde and retrograde direction [83]. Anterograde movement occurs from the basal body to the distal tip of
the cilium and is driven by the IFT complex associating with heterotrimeric or homodimeric kinesin II. Retrograde movement occurs from the distal tip back down to the basal body and is driven by the IFT complex associating with cytoplasmic dynein 2 (Figure 1.1.5b). There are 20 different IFT proteins that have been identified and they are subdivided into two groups, the IFT complex-A and IFT complex-B [84]. They were postulated to function in distinct aspects of IFT since their disruption affects cilia morphology differently. It is now believed that IFT complex-B proteins are involved in anterograde movement while IFT complex-A proteins are involved in retrograde transport (reviewed in Taschner et al. 2012), [85].

In addition to IFT proteins, there is another large protein complex that is also involved in ciliary cargo trafficking called the BBSome. The BBSome is composed of seven BBS proteins, so called because their dysfunction leads to Bardet Biedl Syndrome. They traffic cilia components (other than those that are essential for axoneme formation) and are also involved in vesicle transport from the golgi to the basal body [80].

How specific proteins are targeted to the cilium is still not completely understood, but it has been postulated that selectivity is achieved by a mechanism similar to the one utilized by the nuclear pore. In support of this, components required for nuclear trafficking have been shown to localize to cilia [86]. However, nuclear trafficking requires a nuclear localization signal and while ciliary targeting signals have been identified for some proteins, a clear consensus sequence remains elusive [80, 87].
Figure 1.1.5 Cilia structure. (a-b) The defining feature of the cilium is the axoneme, a long microtubule-derive structure that projects from the basal body. (a) A cross-section of the ciliary axoneme reveals a distinct cartwheel formation of the microtubule array. Outer microtubule doublets are composed of a complete A-tubule and an incomplete B-tubule that exhibit 9-fold symmetry and are connected to each other by nexin links. At the center of the axoneme, there may or may not be a central pair of microtubule singlets that are connected to the doublets through radial spokes, giving the cilium either a 9+0 or 9+2 classification. The microtubule doublets make contact with each other through inner and outer dynein arms, creating a sliding motion that generates cilia beating. (b) The ciliary axoneme is surrounded by the ciliary membrane which is continuous with the plasma membrane, but its composition is distinct. The cilium is compartmentalized from the remainder of the cytoplasm at the transition zone, a region just distal to the basal body where the ciliary necklace and transition fibers limit diffusion into the cilium. Sometimes the cilium resides in the ciliary pocket which is an invagination of the plasma membrane with high endocytic activity. The basal body may also be associated with the basal foot and striated rootlet which are accessory structures that interact with the cytoskeleton. Ciliary outgrowth occurs at the distal end of the basal body and requires IFT to move particles required for cilia formation and maintenance in the anterograde (associated with kinesin-2) and retrograde (associated dynein) direction.
1.1.7 Cilia function

For much of their known existence, cilia have primarily been appreciated for their functions in generating fluid flow. Primary Ciliary Dyskinesia (PCD) is an inherited genetic disorder that is characterized by defects in cilia motility and its clinical manifestations include chronic bronchitis, chronic sinusitis, chronic otitis media, *situs inversus* and infertility. The wide spectrum of defects is due to the fact that motile cilia have been found lining the respiratory epithelia, the ependymal cells of the brain, the female reproductive tract, and even the sperm tail is essentially a modified cilium [88].

*Situs inversus* is a condition where the major visceral organs are reversed and is noteworthy because it highlights the importance of cilia motility during embryonic development. Leftward fluid flow occurring at the embryonic node is required to initiate left-sided gene expression and establish left-right patterning. As described in section 1.1.4, the posterior tilting of basal bodies is required to initiate leftward flow and an artificial reversal of flow was shown to be sufficient to reverse left-sided gene expression in the mouse node [89]. These observations put cilia motility at the heart of an early patterning event in vertebrates.

Recent studies have uncovered a ciliary basis for a long list of devastating genetic disorders, most notably Joubert Syndrome, PKD, and Bardet Biedl Syndrome. Most cilia-related disorders are pleiotropic in nature and are associated with diverse defects that include obesity, blindness, mental retardation, kidney dysfunction, male infertility, and cancer. This phenotypic diversity is due to the fact that all cilia, including motile cilia, are characterized by a spectrum of receptors and ion channels that enable them to detect mechanic, osmotic, photonic, hormonal and olfactory stimuli. This list will likely grow as uncharacterized transmembrane proteins have been shown to localize to cilia and have domains that may function in sensing anything from light to redox state [77].

The first evidence that cilia may be involved in developmental signalling pathways came from a forward genetic screen in the mouse that focused on identifying mutants in Hh signalling. In brief, Hh is a secreted lipoprotein that regulates embryonic patterning and proliferation and has been linked to human birth defects and cancer. Signalling is initiated when Hh ligands bind to the Patched (Ptc) receptor, preventing Ptc inhibition of Smoothened (Smo). Smo is required for
the processing of Glioma (Gli) transcription factors that regulate Hh target genes (reviewed in Figure 1.1.6a and in Eggenschwiler and Anderson 2007), [90]. This screen focused on ENU-induced mutations that disrupt the specification of ventral cell types in the developing neural tube, a phenotype associated with disrupted Hedgehog (Hh) signalling. Surprisingly, two of the identified mutations were shown to disrupt IFT proteins (IFT88 and IFT172) and another disrupted the IFT motor protein, Kif3a. Furthermore, the IFT machinery was found to function downstream of the Ptc receptor and upstream of the Gli targets [91]. Several components of the Hh signalling pathway were shown to localize to the cilium including Smo, Suppressor of Fused (Sufu, a Gli interacting protein that negatively regulates Hh signalling), and the Gli proteins [92, 93]. IFT was shown to be required for Smo localization to the cilium and for the function of both Gli activators and repressors, suggesting that both active and inactive states of Hh signalling require cilia (Figure 1.1.6a), [93-95].

Another developmental pathway that has been shown to require cilia is PDGF signalling. PDGF signalling is active in diverse cell populations and is required for cell migration, proliferation, and survival. PDGF signalling consists of two major ligands, PDGF-A and PDGF-B, which can form homo or heterodimers and bind to either the receptor PDGFR-α or PDGFR-β (reviewed in Satir and Christensen 2007), [77]. In cultured fibroblasts, primary cilia are present when the cells are in their usual state of growth arrest, Go (resting phase), [96]. In situations where proliferation is required, such as during wound healing, stimulation by growth factors leads to ciliary resorption and reentry into the cell cycle. Primary cilia have been shown to be required for growth control in these fibroblasts by regulating PDGF signalling. The receptor PDGFR-α localizes to cilia during growth arrest and when PDGF-AA ligand is added, the ciliary PDGFR-αα is phosphorylated and activated. Activation of PDGFR-αα is followed by the downstream activation of the Akt and Mek1/2-Erk1/2 pathways that also operate in the cilium (Figure 1.1.6b). Examination of fibroblasts derived from Tg737p5pk (ift88) mutant mice revealed that in the absence of cilia, PDGF-AA did not activate PDGFR-αα or the Mek1/2-Erk1/2 pathways [97]. This places cilia at the center of growth control in fibroblasts and thus during embryonic development and tissue remodeling.

The findings that cilia regulate developmental signalling pathways like Hh and PDGF was fascinating and prompted considerable efforts to find additional pathways regulated by cilia. Evidence emerged that cilia promote PCP signalling and modulate the switch between canonical
and noncanonical Wnt (PCP) signalling. The importance of studying the relationship between cilia and PCP signalling is perhaps best highlighted in the context of PKD. PKD is characterized by dilations in collecting tubules that lead to cystic and enlarged kidneys. Normal tubule diameter is thought to be regulated by CE movements and OCD, both processes controlled by PCP signalling. CE movements drive cell intercalations that lead to the lengthening and narrowing of kidney tubules, while OCD ensure that subsequent proliferation occurs in the proper direction [98-100]. However, even though phenotypic analysis suggested that disrupted PCP signalling was the underlying cause of PKD, genetic studies pointed to defects in cilia function.

The connection between cilia and PKD was first established by studying the underlying genetic lesion in the Oak Ridge polycystic kidney (ORPK) mouse mutant. This mutant was used as a mouse model for human autosomal recessive polycystic kidney disease (ARPKD) but the mutated gene, termed $tg73^{orpk}$, was of unknown function. Cloning of the homologous gene in *Chlamydomonas* revealed that $tg73^{orpk}$ encodes IFT88, an IFT complex-B component. IFT88 was required for cilia formation in *Chlamydomonas* and in the mouse kidney and from there, it was postulate that cilia play an important signalling function in the kidney [101]. A model emerged suggesting that cilia within the kidney tubule bend in response to fluid flow, and that this bending causes an intracellular $\text{Ca}^{2+}$ influx. In $tg73^{orpk}$ cells, the flow sensing response is abolished, leading to kidney disease [102]. Cilia sense flow through the mechanosensory transmembrane protein, Polycystin-1 (PKD-1), and the $\text{Ca}^{2+}$ channel, Polycystin-2 (PKD-2), (Figure 1.1.7). Furthermore, PKD-1 and PKD-2 are mutated in autosomal dominant polycystic kidney disease (ADPKD), [103]. Interestingly, a similar mechanism has been shown to operate in the node where PKD-2 and the PKD-1 ortholog, PKD-1L1, are expressed. $\text{Ca}^{2+}$ is elevated on the left side of the node and both PKD-2 and PKD-1L1 are required to establish left-right asymmetry [104].

Since there was convincing evidence that both PCP signalling and cilia are important for PKD, it was proposed that either PCP signalling was required for cilia formation, or that cilia were required for PCP signalling. In the remainder of this introduction, I will focus on how cilia and PCP signalling are connected.
Figure 1.1.6 Cilia are required for Hh and PDGF developmental signalling pathways. (a) When Hedgehog (Hh) ligand is not present (left), Patched inhibits Smoothened (Smo) which is present on intracellular vesicles. Within the ciliary axoneme, Suppressor of Fused (Sufu) processes Glioma (Gli) transcription factors into the repressor forms (Gli-R), which are then transported by retrograde IFT down the cilium and translocate into the nucleus to inhibit the transcription of Hh target genes. When Hh ligand is present (right), it binds to the Patched receptor which alleviates inhibition of Smo. Smo then translocates to the cilium by anterograde IFT, where it prevents the processing of Gli-R by Sufu. This allows Gli activators (Gli-A) to be transported down the cilium by retrograde IFT and to translocate to the nucleus where they activate the transcription of Hh target genes. (b) Proliferating fibroblasts (left) do not have cilia so PDGFR-α is maintained on intracellular vesicles. In quiescent cells (right), PDGFR-α dimerizes (PDGFR-αα) and is phosphorylated and activated. This is followed by the activation of downstream MEK/ERK/Akt signalling and cell cycle entry.
Figure 1.1.7 *Mechanosensory function of cilia in the kidney.* (a) Urine flow through the kidney tubule causes cilia to bend (left). Cilia bending is detected by the mechanosensory transmembrane protein PKD-1, which causes the opening of the associated ion PKD-2 and the influx of Ca$^{2+}$ into the cell. A high concentration of intracellular Ca$^{2+}$ is required to keep a cell in a differentiated state and suppress proliferation. In the absence of cilia bending (right), intracellular Ca$^{2+}$ is decreased, which initiates the transcription of genes that are involved in cell proliferation.
1.2 PCP Signalling and Cilia Formation

1.2.1 Nonspecific regulators of PCP signalling and ciliogenesis

The first indication that PCP signalling may be required for cilia formation came from vertebrate studies of the PCP effector proteins, Inturned and Fuzzy, and later Fritz. In *Drosophila*, these factors have been shown to affect PCP in specific tissues and to be downstream of the core PCP factors. Park et al. (2006) examined the functions of Inturned and Fuzzy using morpholino knockdown in *Xenopus* embryos. *Xint* and *Xfy* morphants showed posterior neural tube closure defects consistent with disrupted PCP-mediated CE movements. Interestingly, defects in anterior neural tube closure were also observed that are not typically observed with disrupted PCP, but are reminiscent of Hh signalling defects. Since cilia had been shown to be required for Hh signalling in the mouse, the investigators examined whether cilia disruption may be the underlying basis of Hh defects in *Xint* and *Xfy* morphant embryos. They found that cilia were severely disrupted in the ventral neural tube and on epidermal cells, and that this resulted from defects in the assembly of apical actin. *Xint* and *Xfy* morphants showed a dense web of microtubules below the apical surface, indicating that Inturned and Fuzzy regulate cytoskeletal organization that is required for the apical orientation of ciliary microtubules [2]. A subsequent study by Park et al. (2008) showed that Inturned functions alongside Dvl to mediate cilia formation by controlling basal body docking at the apical membrane in *Xenopus* multi-ciliated cells of the epidermis. Dvl is required for basal bodies to associate with membrane-bound vesicles and the vesicle trafficking protein Sec8 in order to migrate to the apical membrane. At the apical membrane, Dvl and Inturned are required to activate the Rho GTPase, leading to an enrichment of apical actin that is required for vesicle fusion and normal basal body docking. Once the basal bodies are docked, Dvl and Rho are required to polarize the uniform orientation of basal bodies which is essential for the directional beating of motile cilia on epidermal cells [3]. Analysis of Fritz function in *Xenopus* embryos showed that it too was required for cilia formation but the mechanism differed from that of Inturned and Fuzzy. Fritz knockdown led to fewer and shorter cilia resulting from the disrupted apical positioning of Septin-2 and Septin-7 [105]. Septins are proteins involved in scaffolding and providing structural support and have previously been shown to participate in the diffusion barrier at the base of the ciliary membrane [106].
Saburi et al. (2008) examined the consequences of the loss of Fat4 in mouse embryos. They observed defects that are consistent with disrupted PCP including a shorter body axis, misoriented cochlear stereocilia, and cystic kidneys. Fat4 was found to localize to cilia, suggesting that it may function in cilia formation. However, alterations in cilia number were not observed in most fat4 -/- mutant kidneys but a reduced number of cilia were occasionally observed in kidneys with large cysts [100]. It is unclear whether defects in cilia formation are simply secondary to an abnormal environment in the severely disrupted kidneys.

Based on the above studies, it was established that there was some connection between PCP and cilia formation in vertebrates, but questions still remained. In the case of Inturned, Fuzzy, and Fritz, they were shown to participate in establishing PCP in Drosophila and were shown to be required for cilia outgrowth in vertebrates. Whether their role in cilia formation is a universal feature of PCP signalling is disputed because it is unclear how PCP effectors relate to core PCP signalling. Subsequent studies examining the function of Inturned, Fuzzy and Fritz in mouse embryos revealed that their functions in cilia formation are conserved [107-109]. However, mutants of Intuned and Fuzzy did not exhibit obvious CE defects which are a hallmark of disrupted PCP signalling [108, 109]. Furthermore, neither gene interacted genetically with vangl2 in CE or neural tube formation [110]. While Fritz has been shown to play a role in CE movements, it controls cell shape rather than polarization, indicating that it regulates only a subset of PCP-mediated cell behaviors [105]. The same arguments can be made against the fat4 -/- mutant mouse implicating PCP signalling in cilia formation since it is believed that the Fat-Ds pathway is parallel to the core PCP factors. Also, while Fz-2 and Dvl are considered core PCP factors, their role in cilia formation does not directly implicate PCP signalling. Fz and Dvl are shared with other branches of Wnt signalling like the Wnt β-catenin and Wnt calcium pathways so their function is not specific to PCP signalling [47]. Analysis of core PCP factors that function exclusively in PCP signalling like Celsr, Vang, and Pk, are a more accurate representation of the specific functions of PCP signalling.
1.2.2 Specific regulators of PCP signalling and ciliogenesis

There is some evidence that core and specific PCP factors may also play a role in cilia formation. Tissir et al. (2010) showed that mouse mutants of both Celsr2 and Celsr3 have defects in the formation and planar polarization of cilia in multi-ciliated ependymal cells in the brain. This stemmed from disrupted basal body docking and ultimately led to defective cerebrospinal fluid clearance (CSF) and a buildup of fluid in the brain, termed hydrocephalus. Interestingly, primary cilia in the choroid plexus were normal in these mutants [111]. Also, knockdown of Pk1a in zebrafish embryos led to fewer cilia in KV and laterality defects. However, Pk1a, along with Wnt11, was also shown to regulate the adhesion properties of dorsal forerunner cells which are the precursors to KV. The authors postulated that cilia defects may be secondary to a diminished number of lumen-forming dorsal forerunner cells [112].

Reaching a consensus on the function of Vangl proteins in cilia formation has been more difficult. Initial studies in *Xenopus* embryos using morpholino-mediated knockdown of *vangl2* produced conflicting results in different cell populations. Mitchell et al. (2009) reported a reduced number of apically docked basal bodies in multi-ciliated epidermal cells [113]. In contrast, Antic et al. (2010) reported that nodal mono-cilia were unaffected in the same *vangl2* morphant embryos [58].

Since morpholino studies suffer from inconsistencies and off-target affects, analysis of cilia formation in mutants of Vangl proteins was of great interest, however they too yielded few definitive conclusions. In Chapter 2, I report the study of cilia formation in MZ*vangl2* mutant zebrafish. Analysis of MZ*vangl2* mutants revealed that Vangl2 is not required for cilia formation, but is required for the posterior tilting of motile cilia in Kupffer’s Vesicle (KV), the neural tube, and the pronephric duct. Vangl2 was also shown to control the posterior positioning of motile cilia on the apical surface of floorplate cells. To determine whether positive and negative PCP regulators have differential roles in cilia formation, the function of Knypek was analyzed. MZ*knypek* mutants were not observed to have defects in cilia formation, but did exhibit defects in the tilting and positioning of motile cilia, consistent with what was observed in MZ*vangl2* mutants [114]. The conclusion that Vangl2 is not required for cilia formation was supported by a subsequent study that examined mouse *vangl1/vangl2* double mutants. These
mutants did not have defects in cilia formation in multi-ciliated airway cells, the neural tube, or in cultured MEF cells [115].

There are nonetheless other mutant studies that suggest the contrary. May-Simera et al. (2010) analyzed cilia formation in a different vangl2 mutant allele in zebrafish and reported a reduced number and shorter KV cilia, resulting from defects in basal body docking [116]. The discrepancies between this study and what I reported in Chapter 2 may be explained by the fact that different vangl2 mutant alleles were used. My study utilized the tri\(^{4k50f}\) allele, resulting in a deletion of the open reading frame, while May-Simera (2010) used the tri\(^{m209}\) allele, resulting in a premature termination codon in the last exon [27, 116]. There is evidence that some vangl2 mutant alleles may function as dominant negatives which could explain the cilia formation defects observed with the tri\(^{m209}\) allele (refer to Chapter 4), [117]. One more recent study by Vladar et al. (2012) utilized a conditional mutant allele of vangl1 and reported shorter, sparser cilia in some multi-ciliated airway epithelial cells, suggesting that Vangl1 may participate in cilia formation. Whether this is proof that Vangl1 controls cilia formation is unclear since vangl1CKO\(^{Δ/Δ}\) mutants showed necrosis and had fewer multi-ciliated airway cells [118].

Comparing the various outcomes of studies examining the functions of different core PCP factors, suggests that the role for PCP signalling is likely dependent on the cell type in question. Core PCP signalling may be required for cilia formation in specialized multi-ciliated cells but may only regulate the planar polarity of cilia present on mono-ciliated cells. This is highlighted in Xenopus embryos where vangl2 morphants were observed to have defects in basal body docking in multi-ciliated epidermal cells but had normal nodal cilia [58, 113]. Given the known differences in the biogenesis of basal bodies between mono- and multi-ciliated cells, this is not unlikely. Another possibility is that perhaps PCP signalling is required for the maintenance of cilia, and that different cell populations have varied susceptibilities to ciliary degeneration. Long term affects on cilia maintenance are more difficult to assess because PCP defects lead to embryonic lethality. In support of this, multi-ciliated epidermal cells in vangl1CKO\(^{Δ/Δ}\) mutants had apically docked basal bodies and Dvl was properly localized, despite fewer cilia present [118]. Since the underlying mechanisms required for basal body docking and cilia formation are intact, cilia defects may be the result of their degeneration.
While the role of core PCP signalling in cilia formation is still debatable, it is clear that PCP signalling regulates the planar polarization of diverse populations of cilia. PCP signalling is required for the posterior tilting of nodal cilia, a process essential for achieving a directed nodal flow, and thus initiating the symmetry-breaking event that establishes left-right patterning in vertebrates [58, 59, 114-116]. In the brain, PCP signalling is required for the polarity of cilia on ependymal cells which generates the directed flow of CSF [111]. In addition to preventing hydrocephalus, the beating of ependymal cilia may play additional roles in brain development since CSF flow was shown to establish a gradient of signalling factors required for neuronal migration [119]. Similarly, cilia on epithelial cells lining the mammalian respiratory tract are required for mucociliary clearance which serves as a primary mechanism of defense against respiratory infection [119]. The polarity of motile cilia may still have additional unidentified consequences on vertebrate development and homeostasis. Motile cilia present in the zebrafish neural tube and pronephric duct were shown to have a posterior positioning and tilting [114]. Whether this ciliary polarity and resultant fluid flow plays any role in the function of these organs still needs to be determined.

1.2 Cilia and PCP Signal Transduction

1.3.1 Connections between cilia and PCP

In addition to PCP signalling being required for cilia formation, there is also compelling evidence that cilia may be required for transducing PCP signals. This was brought to light by the analysis of Inversin, a cilia protein that has sequence homology to the Drosophila PCP protein Diego [120]. Mutations in the inversin gene were shown to underlie nephronophthisis type 2, an autosomal recessive cystic kidney disease [121]. Inversin was shown to localize to cilia and inversin knockdown led to defects typically associated with cilia like cystic kidneys and left-right patterning defects [121, 122]. Interestingly, Simons et al. (2005) showed that in addition to ciliary phenotypes, inversin mouse mutants had disrupted hair patterns which are often associated with defects in PCP signalling. In Xenopus, Inversin was shown to be required for CE movements and to interact with both Dvl and Vangl2. Inversin is believed to promote PCP signalling at the expense of canonical Wnt signalling by targeting cytoplasmic Dvl for degradation [8]. Based on this study it was postulated that Inversin, and hence cilia, regulate a crucial switch between the two Wnt signalling cascades.
Cilia were further implicated in regulating PCP-mediated CE movements by the analysis of the mouse bbs4 mutants. Bbs4 is a component of the BBSome, a large multi-protein complex that regulates cilia trafficking and microtubule dynamics. Ross et al. (2005) showed that bbs4 mutants share phenotypes typical of PCP mutants including open eyelids, neural tube closure defects, and disrupted stereociliary bundles in the cochlea. In zebrafish embryos, injecting bbs4 morpholino was shown to exacerbate CE defects in vangl2 mutants, indicating a genetic interaction between Bbs4 and Vangl2. Consistent with the genetic interaction data, Vangl2 was shown to localize to the ciliary axoneme and basal body in mouse cultured kidney cells [7]. A following study found that Bbs1, 4, and 6 were required for CE in zebrafish and that they genetically interact with the noncanonical Wnts, Wnt11 and Wnt5b [5]. Similarly Ofd1, a ciliary protein mutated in Oral-Facial-Digital Type I Syndrome, was shown to be required for cilia formation and CE movements in zebrafish, and CE defects were enhanced by loss of Wnt11 and Vangl2 [4].

Consistent with the idea that cilia control a molecular switch between PCP and canonical Wnt signalling, suppression of Bbs1, 4, and 6 led to the stabilization of cytoplasmic β-catenin and the upregulation of canonical Wnt transcriptional targets. Hyperactive canonical Wnt signalling was due to perturbed proteasomal targeting of cytoplasmic β-catenin [5]. To directly implicate cilia, three separate mutations that disrupt ciliogenesis (affecting Kif3a, IFT88, and Ofd1) were used to study Wnt signalling activity in mouse embryos and in cell culture, and all were found to restrict canonical Wnt signalling [123]. Another protein that was found to link cilia function to the balancing of Wnt signalling pathways is Lrrc6l (seahorse). Studies in zebrafish showed that Lrrc6l was enriched in ciliated tissues and was required to regulate cilia-mediated processes like left-right patterning and preventing kidney cyst formation. Lrrc6l genetically interacts with Inversin and associates with the DEP domain of Dvl. Similar to Inversin, Lrrc6l was shown to promote PCP-mediated CE movements and constrain canonical Wnt signalling, possibly through its physical interaction with Dvl [6].

By integrating all these findings, a model was proposed to explain how primary cilia function in modulating Wnt signalling. Cilia were suggested to act as mechanosensors that respond to a stimulus such as cilia bending, and activate an intracellular signalling response. In the mammalian kidney, cilia may be deflected by the flow of urine over cells lining the nephron tubule, whereas during CE movements, cilia may bend as a result of the movement of
neighboring cells [39, 79]. Bending stimulates the ciliary mechanosensor PKD-1 to trigger the Ca\(^{2+}\) channel PKD-2, leading to an overall increase in the intracellular Ca\(^{2+}\) concentration. This response may be transmitted through Inversin since fluid flow has also been shown to increase Inversin levels [8]. Inversin then binds both Vangl2 and Dvl, but targets only cytoplasmic Dvl for degradation, and consequently acts to repress the canonical Wnt pathway and prevent unnecessary proliferation. Inversin does not inhibit the buildup of membrane-associated Dvl so it can signal through the PCP branch of the Wnt pathway (Figure 1.3.1). The fact that Inversin, Vangl2 and Dvl were all found to localize to cilia or basal bodies indicates that these factors are present in close enough proximity to each other where these interactions are possible [3, 7, 8]. In conditions where cilia do not bend like during kidney injury or when cilia are disrupted, cellular levels of Inversin get reduced. This leads to the stabilization of cytoplasmic Dvl and prevents the destruction of cytoplasmic β-catenin. This ultimately initiates the transcription of canonical Wnt target genes, leading to increased cell proliferation that if unregulated, can lead to the formation of kidney cysts. Consistent with this model, overactive canonical Wnt signalling has been shown to result in cystic kidneys in the mouse [124]. Concomitantly, there is less membrane-associated Dvl available to signal through the PCP pathway which may explain the prevalence of CE defects when cilia are disrupted (reviewed in Singla and Reiter 2006) [79].

While the above model is convenient for interpreting some of the initial studies that implicated cilia in PCP signalling, there are some significant contradictions. Often proteins are deemed to be ciliary because they localize to cilia or basal bodies and are required for aspects of cilia function. However, many of these proteins localize to other areas of the cell as well, where they may serve other non-ciliary functions. Inversin for example, was shown to localize to cell junctions and the nucleus in addition to its ciliary localization [125, 126]. Since these proteins localize to non-ciliary compartments, observed PCP defects cannot be directly linked to cilia function. In the case of BBS proteins, they have been shown to regulate microtubule dynamics throughout the cell and oriented microtubules have been shown to asymmetrically localize PCP determinants [127, 128]. Defects in PCP-mediated CE movements observed when BBS proteins are knocked-down may actually be related to their functions in orienting cytoplasmic microtubules. The strongest argument against a role for cilia in transducing PCP signals is that mutants of IFT proteins, traditionally believed to function exclusively in cilia formation, were not reported to exhibit PCP-mediated CE defects.
Figure 1.3.1 The role of cilia in modulating Wnt signalling. Cilia may bend in response to different extracellular forces like fluid flow or the movement of neighboring cells during CE movements (left). Bending is sensed by the mechanosensor PKD-1 which causes the influx of Ca^{+2}, mediated by the PKD-2 ion channel. The influx of intracellular Ca^{+2} upregulates the expression of Inversin, which acts as a switch between the canonical (β-catenin) and non-canonical (PCP) Wnt signalling pathways. Inversin targets cytoplasmic Dvl for degradation, but leaves membrane-associated Dvl unaffected, thus specifically inhibiting canonical Wnt signalling while enabling PCP signalling to proceed. When cilia bending is prevented (right), there is no influx of intracellular Ca^{+2}, causing Inversin levels to be reduced. This alleviates the degradation of cytoplasmic Dvl, enabling it to signal through the canonical Wnt pathway.
1.3.2 IFT proteins and PCP

Analysis of mouse mutants of IFT proteins showed that IFT was required for cilia formation, but also revealed defects that may be associated with disrupted PCP. Mouse Tg737Δ2-3βGal (ift88) mutants had shorter cilia which led to ciliary defects like kidney cysts and laterality defects, but were also observed to have neural tube closure defects [129]. The neural tube defects were believed to resemble those arising from disrupted Hh signalling more than those arising from disrupted CE movements, but it is possible that they could be masked by the Hh defects. IFT88 inactivation was also shown to cause stereociliary bundle misorientation on cochlear cells, a defect typically observed in PCP mutants. IFT88 was also shown to genetically interact with Vangl2, but core PCP proteins were found to partition normally in ift88 mutants [130]. It may be that both cilia and core PCP factors play some role in polarizing stereociliary bundles, but it does not appear that IFT88 is required to directly transmit PCP signals in the cochlea.

Zebrafish IFT mutants were not found to have a shorter and broader body axis, indicative of CE defects. However, studying the function of IFT proteins in zebrafish is difficult because the genes are expressed maternally. This maternally deposited RNA and protein enables IFT mutants to form cilia, but not maintain them. In the case of the ift88 (oval) mutant, cilia persist till approximately 4 days post fertilization [131]. This means that cilia are still present during late gastrulation, which is when the bulk of PCP-mediated CE movements occur.

Ocbina et al. (2009) attempted to directly study the function of cilia in modulating Wnt signalling by doing a systematic analysis of mouse embryos lacking IFT88, IFT172, Kif3a, and Dyn2h1. They assessed canonical Wnt signalling activity using the expression of the direct target axin2 and the activation of a transgenic canonical Wnt reporter. Both were found to be expressed in their normal spatial pattern and to the same quantitative level in mutants. They also examined the switch between canonical and noncanonical Wnt (PCP) signalling by testing the ability of a noncanonical Wnt ligand to block the responsiveness to a canonical Wnt, and found that the switch between the two branches was normal [132]. In contrast, a later study by McDermott et al. (2010) analyzed mouse mammary epithelial tissue of Tg737opk (ift88) mutants and found an increase in canonical Wnt signalling with decreased Hh signalling. These changes led to defects in branching morphogenesis and implicated cilia in mammary gland development [133]. A study
in zebrafish by Cao et al. (2010) suggested that IFT proteins do in fact regulate PCP signalling because basal bodies in multi-ciliated kidney cells were disorganized in *ift57* and *ift172* mutants. They argued that this was a defect in PCP signalling because a similar disorganization was observed by depleting Pk1 and because Pk1 genetically interacted with the IFT genes [134]. Since CE defects are a hallmark of disrupted PCP signalling in zebrafish, their absence in IFT mutants still argued against a role for cilia in PCP signalling. However, since cilia were still present for much of early development in zebrafish IFT mutants, the role for cilia in these processes remained unclear.

In order to eliminate maternal contribution and disrupt cilia formation from the onset of zebrafish development, Huang et al. (2010) generated MZ*ift88* mutants. These mutants were found to have defects in initial cilia formation where the ciliary axoneme appeared absent but they developed a normal body axis, suggesting that CE movements are occurring normally. MZ*ift88* embryos did not exhibit any morphological abnormalities resembling canonical Wnt signalling mutants and the expression of Wnt target genes was normal. A defect in Hh signalling was observed but it differed from the mouse defect where instead of being simply down regulated, the expression domain was dampened, but expanded [135]. In Chapter 3, I also report the generation of MZ*ift88* mutants and a thorough analysis of PCP signalling in these mutants revealed normal CE of the neural tube and somites, normal migration of the prechordal plate, and normal extension of the body axis during somitogenesis. Analysis of MZ*ift88* mutants strongly suggests that the ciliary axoneme is not required for PCP signalling. If there is any role for cilia in regulating the switch between PCP signalling and canonical Wnt signalling, it is probably very subtle and is buffered in the embryo. Alternatively, these functions may be cell type specific and occur only at certain developmental time points. Transmission electron microscopy revealed that while the axoneme is largely absent in MZ*ift88* mutants, they maintained properly docked basal bodies. While the mechanosensory function of cilia is eliminated, the basal body function is likely left intact, and it is possible that the basal body could mediate PCP signalling in the absence of the axoneme.

1.3.3 Non-cilia functions of IFT proteins
Interestingly, analysis of MZ*ift88* mutants revealed that IFT88 was required for OCD occurring during zebrafish gastrulation (refer to Chapter 3). It was previously postulated that during interface, sensory inputs to the cilium might get transduced to the basal body, and that this in
turn might bias the position of the spindle during cell division. In the case of OCD occurring at gastrulation, this is not possible because they precede initial cilia formation [114]. This suggests that the role IFT88 plays in OCD during gastrulation must be cilia independent. Traditionally, IFT proteins have been thought to localize to and function exclusively in cilia formation and maintenance. The only exception was considered to be IFT20, which was known to localize to Golgi and secretory vesicles and functions in the targeted delivery of cilia proteins [136]. There is increasing evidence that this is also true for other IFT proteins. IFT88 was shown to regulate G1-S transition during cell cycle progression in non-ciliated HeLa cells [137]. During cell division, IFT88 was also implicated in astral microtubule formation by participating in a dynein1-driven complex. This complex is required for transporting microtubule-nucleating proteins and peripheral microtubules clusters to spindles [138]. Moreover, IFT proteins are expressed in non-ciliated lymphoid and myeloid cells where IFT20, IFT57, and IFT88 were found to form a complex. This complex associates with the T-cell receptor (TCR/CD3) and is required for signalling at the immune synapse and TCR clustering at the membrane [139]. This suggests that IFT proteins may play a more general role in polarized membrane transport which may require us to reexamine some of the functions that have been attributed to cilia.

1.3.4 An indirect role for cilia in regulating PCP

An elegant study by Mitchell et al. (2007) was the first to show that a positive feedback loop is required to establish the polarity of motile cilia on multi-ciliated Xenopus epithelial cells [140]. These cells exhibit a uniform posterior orientation of basal bodies/cilia within an individual cell, as well as a tissue wide coordination between neighboring cells. First, cilia orientation is roughly set by a tissue-patterning phase that requires PCP signalling non-cell-autonomously [113]. This is followed by the refinement phase which occurs after gastrulation and requires the fluid flow generated by the roughly oriented cilia themselves. This fluid flow was shown to be both necessary and sufficient for defining cilia orientation, suggesting that motile cilia could play an indirect role in affecting the PCP of certain cells [140].

In mammals, a similar mechanism operates in multi-ciliated mouse brain ependymal cells, where basal bodies first dock with random orientation and then reorient in a common direction. Guirao et al. (2010) showed that the orientation of basal bodies/cilia is established by a combination of hydrodynamic forces generated by motile cilia and intracellular PCP. External fluid flow applied
over ependymal cells during maturation was shown to orient cilia in the direction of fluid flow. In contrast to what was observed in *Xenopus* epithelial cells, external fluid flow was not sufficient to define cilia orientation, and this process required Vangl2. This suggests that the role cilia play in the establishment of ependymal PCP is not simply downstream of PCP signalling [141].

To determine whether the same mechanisms operate in mono-ciliated cells, I examined the polarity of basal bodies/cilia on floorplate cells in the zebrafish *MZift88* mutants. Floorplate cilia exhibit two types read-outs of PCP, where basal bodies dock at the posterior surface of the apical membrane, and are oriented in the posterior direction [114]. Since *MZift88* mutants have defects in axoneme formation, it is an ideal model to study basal body/cilia polarity in the absence of cilia-generated fluid flow. Because basal bodies remain docked at the apical membrane of *MZift88* mutants, it is possible to assess their position and orientation. In Chapter 3, I report that the posterior positioning of basal bodies at the apical surface of floorplate cells is normal in *MZift88* mutants, suggesting that the positioning of primary motile cilia does not require cilia-generated fluid flow. I have some evidence that fluid flow may be required for the orientation of basal bodies/cilia. WT donor cells labelled with the cilia marker Arl13b-GFP were transplanted into the neural tube of unlabelled *MZift88* hosts and cilia orientation was assessed. In one instance, the cilium of a labelled WT cell was observed to sway when transplanted into a *MZift88* mutant background. This was curious because swaying was never observed when WT cells were transplanted into WT hosts, and this may suggest that fluid flow is required to reinforce cilia orientation. However, due to the technical challenges of this experiment, sufficient numbers were not obtained to make definitive conclusions. Clearly this topic requires further examination and may be aided by the study of basal body accessory structures like the striated rootlet, which have previously been used to determine basal body orientation [3].

### 1.4 Thesis Aims

In light of all the evidence linking cilia and PCP signalling, a focus of my thesis has been to gain a better understanding of how the two are related. Specifically, my aims have been to conclusively determine whether core PCP signalling is required for cilia formation, and whether cilia are required to regulate PCP. In the first part of my thesis (Chapter 2), I report the analysis
of cilia formation and function in zebrafish MZvangl2 mutants, complete loss of function mutants of a core and specific PCP regulator. In Chapter 3, I report the generation and analysis of MZifi88 mutants that lack all cilia formation, which I utilized to determine the role that cilia play in regulating PCP.
Chapter 2

2. Investigating the role of core PCP factors on cilia formation and function

The data presented in this chapter was published in 2010 in:

2.1 Abstract

Cilia have important roles in maintaining homeostasis and in regulating developmental signalling pathways, and their dysfunction has been associated with diverse human diseases. Recent studies have suggested that PCP signalling is required for initial cilia formation. However, this assumption is primarily based on studies of PCP factors such as Inturned, Fuzzy and Dvl, whose function is not specific to PCP signalling [2, 3]. To determine the role of a specific PCP factor on cilia formation, I analyzed a complete lose of function vangl2 mutant in zebrafish. Cilia were visualized using Arl13b-GFP labelling that enables the study of cilia structure and dynamics in living embryos. Analysis of ciliogenesis revealed that Vangl2 is not required for cilia formation, but is required for controlling the posterior tilting of motile primary cilia present in KV, the ventral neural tube, and the pronephric duct. I also show that in addition to controlling the tilting of motile cilia, Vangl2 also controls the posterior positioning of motile cilia on the apical surface of floorplate cells. Subsequent analysis of the function of Knypek, another regulator of PCP signalling, showed that it was not required for cilia formation. It too was required for motile primary cilia tilting and positioning, indicating this is a function shared with other PCP factors.
2.2 Brief Introduction and Rationale

The PCP pathway was first discovered in *Drosophila* and it enables diverse tissues to orient their cells in a plane (perpendicular to AB polarity). In vertebrates, the PCP pathway is also referred to as non-canonical Wnt signalling because the ligands for the pathway are Wnts, but β-catenin is not stabilized, which is a hallmark of canonical Wnt signalling [47]. PCP is initiated by *core PCP factors* that control polarity in all planar polarized tissues, and *cell specific effectors* that control polarity only in some contexts and do not affect the distribution of the core factors [22]. Core PCP factors are asymmetrically localized and influence polarity by acting on the cytoskeleton [16]. They are further classified as positive regulators like Wnts and Fz, that induce cytoskeletal rearrangements in cells, and negative regulators like Vangl2 and Pk, that restrict them to one side of the cell [16-18]. In vertebrates, PCP signalling has been shown to regulate polarized CE movements and OCD, resulting in the lengthening and narrowing of the body axis [25]. Recent studies have also implicated PCP signalling in the formation of cilia, which has generated great interest in the field [2, 3, 113].

The most compelling evidence that PCP signalling regulates ciliogenesis comes from studies of multi-ciliated epithelial cells in *Xenopus*. Park et al. (2006) knocked down the PCP effectors Inturned and Fuzzy using morpholinos (MO) and found that this not only caused PCP defects, but also Hh defects which resulted from disrupted ciliogenesis. The authors noted defects in apical actin localization and argued that this perturbed basal body docking, which led to the formation of cytoplasmic cilia [2]. Park et al. (2008) subsequently showed that Dvl was also required for the apical docking of basal bodies and the directional beating of motile cilia [3]. Mitchell et al. (2009) performed MO-mediated knockdown Vangl2 in the same multi-ciliated cells and also reported defects in cilia formation [113].

At the time of this study, no mutants of core PCP proteins have been reported to have defects in motile or primary cilia formation. Many of the proteins analyzed like Inturned and Fuzzy are considered PCP effectors and it is unclear how these proteins fit in with core PCP signalling, or if they have roles outside of regulating PCP signalling. While Dvl is considered a core PCP factor, it is also shared with other branches of Wnt signalling, so a direct correlation between cilia formation and PCP signalling cannot be made. Moreover, when Park et al. (2008) injected Xdd1, a mutant Dvl construct that is a well-established dominant negative inhibitor of PCP, they
did not observe defects in cilia formation [3]. Of the core PCP factors, only Vangl2 was implicated in cilia formation and this study relied on MO-mediated knockdown, which is notorious for causing off-target affects [113]. The lack of clear data on cilia formation in PCP mutants may be due to the fact that many of these proteins have redundancies (eg. Vangl1 and Vangl2) and therefore the presence of other paralogs may mask mutant phenotypes [24]. Another issue is that core PCP proteins are expressed maternally. Maternal gene products from heterozygote mothers may mask early aspects of the mutant phenotype in homozygous mutant progeny [142]. In which case, studying the zygotic mutant may not be appropriate for determining the functions of proteins in early development, and may require the analysis of MZ mutants.

My objective was to study cilia formation in a mutant of a core PCP factor and I chose Vangl2 because it functions exclusively in PCP signalling. Vangl2 is a four-pass transmembrane protein that restricts the membrane localization of Dvl [17, 18]. The zebrafish vangl2 mutant (trilobite) has been shown to have both cell-autonomous and non-cell-autonomous CE defects during gastrulation and neurulation [27, 43]. The vangl2 mutant was subsequently shown to have significant maternal contribution and the MZ mutant was revealed to have more severe CE defects than the zygotic mutant, but cilia were not studied [43].

Here, I report the complete analysis of cilia formation in MZvangl2 mutants. I show that Vangl2 is not required for cilia formation, but is required for the posterior tilting and positioning of primary motile cilia.
2.3 Materials and Methods

2.3.1 Zebrafish strains

Established zebrafish husbandry protocols were adhered to for all strains used throughout the duration of the study, and all protocols were approved by the Animal Care Committee at the Hospital for Sick Children. The *tri*(*tk50f*) *vangl2* mutant allele was chemically induced, containing a deletion in the coding sequence and as a result, transcripts of *vangl2* are not detected in *tri*(*tk50f*) homozygous mutant embryos [27]. Chimeric zebrafish harboring a homozygous *vangl2* mutant germline were generated according to a previously published germline replacement strategy [143]. Mating of adult *vangl2* mutant chimeras was used to obtain MZ*vangl2* embryos. MZknypek mutant embryos were generated from the *kny*(m119) mutant allele using the same germline replacement strategy. This allele is also chemically induced and consists of a base pair change that leads to a premature stop codon [50]. The mouse Arl13b open reading frame was gifted to us by Tamara Caspary (Emory University, Atlanta, GA) and was used to generate Tg(βact::Arl13b-GFP/Cherry) fish. The zebrafish Tol2kit was used to assemble Gateway compatible vectors containing fluorescently tagged Arl13b under the control of the β-actin promoter. Transgenic vectors were injected into one-cell stage embryos and incorporated into the genome using Tol2-mediated transposition. The injected “founders” were grown-up and crossed to WT fish and their progeny were screened for the presence of red or green fluorescence in the progeny, indicating germ-line transposition in the founder population.

2.3.2 Embryo microinjections

Plasmids encoding the open reading frames of desired genes were linearized and the mMESSAGE mMACHINE system (Ambion) was used for the *in vitro* synthesis of mRNA. The following concentrations of mRNA were injected into one-cell stage embryos for *in vivo* localization studies: 20pg of membrane-localized monomeric red fluorescent protein (memb-mRFP), 200pg of EGFP-tagged *Drosophila* Prickle (GFP-Pk), 2.5pg of GFP-tagged *Xenopus* Centrin (Cen-GFP; Adrian Salic, Harvard University, Boston), and 7pg of Arl13b-GFP/Cherry. Injection of 300pg of WT zebrafish *vangl2* mRNA was used for over-expression analyses. MOs were obtained from Gene Tools, LCC, and were designed and diluted according to instructions provided by the manufacturer. A MO targeted against the 5’UTR of *vangl1* (v1MOa; 5’
CATGGCAATGGCGTCTGTGCTG) was injected into one-cell stage embryos at 2-6ng to block the translation of *vangl1* mRNA. Additionally, a MO was designed to target the exon 5/ intron 5-6 boundary of *vangl1* (v1MOb; 5'TCACCATCCACATTAGACTTTCA) and thus prevent splicing of *vangl1* pre-mRNA and was injected at 7 or 14ng.

### 2.3.3 Cell transplantation techniques

Cell transplants were utilized to achieve chimeric labelling and to study cell autonomy. Transplants were performed using established techniques at mid-blastula stages (4hpf), since cells are multi-potent and adhere to each other less tightly at this stage, making transplants easier. Either WT or MZ*vangl2* donor embryos were injected with Arl13b-GFP and memb-mRFP to label cilia and membranes. Approximately 50-100 cells were transplanted from donor embryos into a region just dorsal to the embryonic margin of unlabelled WT host embryos. Transplanting into this region increases the likelihood that donor cells contribute to neuroepithelial cells lineages.

### 2.3.4 Reverse transcriptase (RT) - PCR assay

We performed a RT PCR strategy to determine the efficacy of the splice-blocking *vangl1* MO (v1MOb). One-cell stage WT embryos were either injected with 7 or 14ng of v1MOb or were left as uninjected controls and approximately 50-100 embryos from each subset were collected at the 21-somite stage (19.5hpf). Total RNA was extracted from each subset using TRIzol reagent (Invitrogen) before SuperScript II reverse transcriptase (Invitrogen) and Oligo (dT) 12-18 primer (Invitrogen) were used to generate first-strand cDNA from the RNA samples, all according to manufacturers’ instructions. The v1MOb was designed to block the exon-intron boundary of exon 5 so in order to test the efficacy of this MO, we used three different primers to detect abnormal splicing events. A forward primer was designed to span exon 3 (For: 5'TATACGCTTCTTCCGTTTG) and reverse primers that span either intron 5 (Rev1: 5'GGGAGAAGCAGTTCATTTGC) or exon 8 (Rev2: 5'GAGATCAACGCACCTCCATCA) were used. Phusion High Fidelity DNA Polymerase (NEB) was used to PCR amplify from all three primers using cDNA from WT, 7ng MO-injected, or 14ng MO-injected embryos with the following PCR conditions: 98°C for 30s, 35 cycles of 98°C for 10s, 63°C for 30s, 72°C for 45s, followed by 72°C for 10 min.
2.3.5 Whole mount RNA *in situ* hybridization

To assess left-right patterning, *lefty-2* and *lefty-1* riboprobes were generated from linearized DNA plasmids by *in vitro* transcription using a DIG RNA Labelling Kit (Roche), digoxigenin-11-UTP (Roche), and T3 polymerase (Roche). Standard protocols for whole-mount RNA *in situ* hybridization of zebrafish embryos were used. Embryos were subsequently cleared in 100% methanol and mounted in benzylbenzoate/benzylalcohol (2:1) prior to imaging on an Axio Imager.M1 compound microscope (Zeiss).

2.3.6 Immunohistochemistry (IHC)

For IHC, embryos were fixed at appropriate stages using 4% PFA overnight at 4°C. Embryos were dehydrated in 100% methanol followed by stepwise rehydration and were rinsed in PBS + 1% Tween-20. Blocking was done in PBDT (0.1% Tween-20 + 1% DMSO in 1X PBS) + 10% normal goat serum for 2 hours at room temperature. The following primary antibodies were used: mouse anti-acetylated α-tubulin (1:200, Sigma), rabbit anti-GFP (1:500, Invitrogen), and were applied overnight at 4°C. PBDT + 1% normal goat serum + 0.05M NaCl was used for all washes, before and after addition of appropriate Alexa Fluor-conjugated secondary antibodies (1:500, Invitrogen). SlowFade glycerol solution (Invitrogen) was used to clear embryos before they were mounted for imaging using a Zeiss 710 laser scanning confocal microscope.

2.3.7 Analysis of KV size and flow

To study flow within KV, 8-somite stage WT and MZ*vangl2* embryos were mounted in 0.8% low melt agarose with KV facing upward (away from coverslip). Red fluorescent beads (0.5µm, Polysciences Inc.) were diluted 20-fold in Danieau’s solution and one drop (0.5nL) was carefully injected into KV using a glass microinjection needle. An Axio Imager.M1 (Zeiss) compound microscope with a 40X water objective was used to image KV and measure diameter. Time-lapse videos of the flow of fluorescent beads were captured with an AxioCam HRm (Zeiss) camera and AxioVision 4.7 (Zeiss) imaging software. Z-stack projections were made from time-lapse movies using ImageJ 1.42q software in order to trace bead trajectories.
2.3.8 *In vivo* analysis of cilia form and function

Either Arl13b-GFP/Cherry mRNA-injected or Tg(ßact::Arl13b-GFP/Cherry) embryos were used for the *in vivo* study of various populations of cilia. Embryos were mounted on a coverslip in 0.8% agarose and imaged using a Zeiss 710 laser scanning confocal microscope. The slower acquisition speeds and image averaging of laser scanning microscopy gave motile cilia a fan-like appearance. The fan-like appearance of motile cilia meant that in addition to cilia length, I could also measure their orientation and range of motion using Zeiss ZEN 2009 imaging software. The angle between the extreme ends of the fan-like cilia was assumed to be the range of ciliary motion. The orientation of motile cilia was measured by determining the angle between the central axis of ciliary motion and the posterior surface of the apical cell membrane. When necessary, Z-stack projections were generated from confocal sections using ImageJ software. The Quorum spinning disk confocal microscope (Zeiss) and Volocity acquisition software (Improvision) were used for high speed imaging of cilia dynamics and enabled time-series to be collected at 27 frames per second.

2.3.9 Statistical analysis

Statistical analysis comparing the tilting of motile cilia in the ventral neural tube in WT and MZvangl2 embryos was done using a two-tailed T-test. Since the angles can only range from 0-180 degrees, circular statistics do not apply and the angle can be considered a linear variable. Our data were in agreement with the Central Limit Theorem since our sample size was large enough and the sampling distribution approached normal and statistical significance was assigned when the P-value was less than 0.05 (alpha). Statistical analysis comparing the positioning of basal bodies at the apical surface of floorplate cells in WT, MZvangl2, MZvangl2+v1MO, vangl2 OE, and MZknypek embryos was compared using a Chi-square test. This test was appropriate since basal body position was classified as anterior, central or posterior and thus, the data represented discrete independent variables. A stricter alpha level of 0.005 was set to determine statistical significance because multiple comparisons were made on the same data set, which could lead to an inflated Type I error rate.
2.4 Results

2.4.1 MZvangl2 mutants have defects in left-right asymmetry and KV fluid flow but have normal cilia formation

MZvangl2 mutant embryos were generated through a germline replacement strategy as previously published [43]. These mutants are a complete loss of function since both maternal and zygotic vangl2 gene product is completely eliminated. Previous work in the lab suggested that MZvangl2 mutants have defects in left-right patterning, which are often associated with cilia dysfunction. Motile cilia lining the mouse node are required to generate leftward fluid flow that initiates a transcriptional response on the left side of the developing embryo, through mechanisms that are not fully understood [144]. A similar system operates within KV in zebrafish, where motile cilia establish an overall counter-clockwise flow within the enclosed vesicle [70]. To further examine laterality in MZvangl2 mutants, a fellow student (Simone Superina) studied lefty-2 gene expression in the lateral plate mesoderm using RNA in situ hybridization [145]. While in WT embryos lefty-2 is typically expressed on the left side of the lateral plate mesoderm, MZvangl2 embryos had a significant increase in bilateral and right-sided expression (Figure 2.4.1a). These defects were not observed in vangl2 +/- embryos or zygotic vangl2 mutants, underscoring the importance of studying a complete loss of function mutant (Figure 2.4.1a).

Since disrupted left-right patterning can result from defects in various downstream mechanisms that are unrelated to cilia function, I proceeded to directly analyze cilia-generated fluid flow within KV [146]. I injected small fluorescent beads into the KV lumen of 8-10 somite-stage embryos and imaged KV from the dorsal side using both DIC and fluorescent video-microscopy, following the trajectories of individual beads. In agreement with published reports, in a majority of WT embryos, there was an overall counterclockwise flow (n=14/15; Figure 2.4.1b). Flow within KV of MZvangl2 mutants was disrupted and local irregularities in bead trajectories were observed (n=4/10; Figure 2.4.1c). These results suggest that defects in left-right patterning in MZvangl2 mutants may result from disrupted cilia-generated fluid flow within KV.

To directly study cilia formation in MZvangl2 mutants, I performed IHC on 6-8 somite-stage embryos using an antibody against acetylated α-tubulin to label stable cilia in KV. Surprisingly, I
found that cilia formation is not affected in MZvangl2 mutants when compared to WT embryos, despite defects in KV fluid flow (Figure 2.4.1d,e).

2.4.2 An expanded KV is not the basis for left-right patterning defects in MZvangl2 mutants

I consistently observed that the diameter of KV is larger in MZvangl2 mutants (73.4µm ± 11.7; n=11) than in WT embryos (58.2µm ± 9.9; n=12), which may result from severely disrupted CE movements in mutants (Figure 2.4.2a,c,d). An enlarged KV could potentially affect fluid dynamics within KV and this could explain the presence of left-right patterning defects in MZvangl2 mutants. Contrary to this, zygotic vangl2 mutants also have an enlarged KV (75.7µm ± 5.9; n=13), but did not exhibit laterality defects (Figure 2.4.2a,b,d). This suggests that the left-right patterning defects observed in MZvangl2 mutants are not due to an expanded KV.
Figure 2.4.1. **MZvangl2 mutants have defects in left-right patterning and KV fluid flow but have normal cilia formation.** (a) Percentage of embryos that showed left (normal), right, bilateral (bilat), and absent *lefty2* expression in 20-22 somite stage wild-type (WT), *vangl2* heterozygote (+/-), *vangl2* mutant (v2), and MZvangl2 mutant (MZv2) embryos. (b,c) DIC images (left) and Z-stack projection of fluorescent time-lapse images (right) of WT (b) and MZvangl2 (c) KVs injected with fluorescent beads at the 8-10 somite stage. Representative bead trajectories have been indicated. (d,e) Immunohistochemical analysis of the KV of WT (d) and MZvangl2 mutant (e) embryos. Anti-acetylated-tubulin staining (AcTub) is shown in red and KVs were imaged from the dorsal side, and Z-stack confocal projections are shown. Cilia formation appears normal in the KV of MZvangl2 mutant embryos (e).
Figure 2.4.2. An expanded KV is not the basis for left-right patterning defects in MZ\textit{vangl2} mutants. (a-c) DIC images of WT (a), \textit{vangl2} mutant (b), and MZ\textit{vangl2} mutant (c) KVs taken at approximately the 8 somite stage, and imaged from the dorsal side. (d) Quantification of the mean and standard deviation of KV diameter reveals a similar increase in KV size for both \textit{vangl2} and MZ\textit{vangl2} mutants. However, zygotic \textit{vangl2} mutant embryos do not demonstrate left-right patterning defects.
2.4.3 Arl13b-GFP is a faithful *in vivo* marker of cilia and it co-localizes with acetylated α-tubulin

Another possible explanation for the presence of left-right patterning defects in MZvangl2 mutants is that while cilia are still present in MZvangl2 mutants, they may not be positioned or oriented properly. In the mouse node it has already been established that since the motile cilia have a clockwise rotational motion, their posterior tilting is required to achieve a unidirectional leftward flow [147]. In KV, the resultant flow is counterclockwise, with cilia reported to be positioned and tilted posteriorly [148]. However, a detailed analysis of cilia function and orientation within KV is limited since the fixation involved in labelling cilia using IHC disrupts overall KV morphology. The lack of alternative methods to label cilia has been a great hindrance, underscoring a need for a live marker of cilia.

In an attempt to generate an *in vivo* marker of cilia form and function, I began to test the subcellular localization of fluorescently-tagged cilia proteins and examine their ability to label cilia. Arl13b is a member of the Arl family of small GTPases that regulate membrane trafficking and was found to be ideal for this purpose. Arl13b was previously shown to localize along the axoneme in mouse embryonic fibroblasts, the mouse neural tube, and the E 8.0 node, and be required for cilia formation in the mouse mutant hennin and the zebrafish scorpion mutant [149, 150]. I injected embryos with mRNA encoding fluorescently-tagged murine Arl13b constructs and imaged ciliated organs at various stages of zebrafish development using laser scanning confocal microscopy. Arl13b-GFP localized to primary cilia in all tissues examined including the ectoderm, notochord, otic vesicle and somites (Figure 2.4.3a-d). Importantly, a time course of early embryogenesis revealed that ciliogenesis begins during late gastrulation (75-80% epiboly; 8hpf), (Figure 2.4.3a). This is not surprising, given that early zebrafish development is typified by rapid and synchronous cell divisions [151]. Since cilia either disassemble or are not present during cell division, the lengthening of the cell cycle that that begins during the mid-blastula transition (3hpf), may somehow initiate cilia formation.

To ensure that Arl13b-GFP was faithfully labelling cilia, I performed IHC to test whether Arl13b-GFP co-localized with acetylated α-tubulin. Co-localization was observed in motile cilia lining KV and the floorplate of the neural tube in both WT and MZvangl2 embryos, suggesting that Arl13b-GFP could be used as an alternative cilia marker (Figure 2.4.4a-d). To fully realize
the benefits of an *in vivo* marker, I generated stable transgenic lines expressing Arl13b under the strong, ubiquitous β–actin promoter [Tg(βact::Arl13b-GFP)].
Figure 2.4.3. Arl13b-GFP expression enables live imaging of cilia form and function. (a-d) Confocal images of WT embryos expressing the ciliary axoneme marker Arl13b-GFP (green) and membrane-localized mRFP (red). (a) Lateral view of the mesodermal germ layer at late gastrulation demonstrating the first appearance of primary cilia at 75-80% epiboly. Interestingly, primary cilia formation is commensurate with the initiation of PCP-mediated convergence and extension movements. (b) Coronal section through the notochord and adjacent somites of a 10-somite stage embryo. (c) The otic vesicle at 30 hpf. (d) Sagittal view of primary cilia in the tail somites at 30hpf. som, somites; not, notochord.
Figure 2.4.4. Arl13b-GFP and acetylated tubulin colocalize along the axoneme of primary motile cilia. (a-d) Immunohistochemical analysis of KV (a,b) and the floorplate (c,d) in WT (a,c) and MZvangl2 mutant (b,d) embryos that express Arl13b-GFP. Anti-acetylated-tubulin staining (AcTub) is shown in red, and anti-GFP staining (Arl13b-GFP) is shown in green. Acetylated-tubulin and Arl13b-GFP co-localize to the ciliary axoneme.
2.4.4 MZvangl2 mutants have defects in cilia orientation within KV

Using our live cilia marker, I reexamined cilia in KV by injecting Arl13b-GFP RNA into one cell stage WT and MZvangl2 embryos and imaged their KV at 6-8 somites. Imaging of KV in WT embryos revealed motile cilia that had a fan-like appearance due to the slower scan speed and image averaging of laser scanning confocal microscopy (Figure 2.4.5a). Interestingly, I also observed the presence of immotile cilia which may serve a sensory function (arrow; Figure 2.4.5a). These observations are consistent with a model previously proposed that suggested that two populations of cilia are required to establish left-right patterning. The model states that in the node, motile cilia are required to generate asymmetric fluid flow, and that this fluid flow leads to the activation of mechanosensory cilia on one side of the node and initiates Ca\textsuperscript{+2}-mediated signalling [152]. In KV of MZvangl2 mutants, I also identified the presence of motile and immotile cilia (Figure 2.4.5b). I utilized the fan-like appearance of motile cilia to measure their average length and range of motion and found no difference between WT (4.8 ± 0.8µm, n=82; 50.0 ± 9.4°, n=81) and MZvangl2 (4.8 ± 0.8µm, n=82; 51.4 ± 9.4°, n=82) cilia (Table 2.4.1).

To determine whether cilia orientation is disrupted in MZvangl2 mutants, z-stack projections were generated from the roof to the floor of KV and cilia orientation was assessed (Simone Superina). With the exception of cilia lining the posterior wall of KV (red arrows, Figure 2.4.5c), the majority of WT cilia projected posteriorly (86% n=50; yellow arrows, Figure 2.4.5c), consistent with previously reported observations [148]. In contrast, z-stack projections through MZvangl2 KV revealed that only 49% of these cilia tilted in the posterior direction (n=57; yellow arrows, Figure 2.4.5d). MZvangl2 mutants also had some cilia projecting out of the plane (purple arrows, Figure 2.4.5d), and also adjacent cilia oriented in opposite directions (circles, Figure 2.4.5d). These observations indicate that Vangl2 is required for cilia orientation, suggesting that defects in cilia orientation may underlie the aberrant KV flow observed in MZvangl2 mutants.
Figure 2.4.5. MZvangl2 mutants have defects in cilia orientation within KV. (a,b) Confocal images through the KV wall of 6-8 somite-stage WT (a) and MZvangl2 (b) embryos that express membrane-localized mRFP (red) and Arl13b-GFP (green) demonstrate the presence of both motile and non-motile (arrows) cilia. Anterior is up. (c,d) Z-stack confocal projections (left) through the floor and wall of Arl13b-GFP (green) labeled WT (c) and MZvangl2 (d) KV, together with a schematic representation of cilia orientation (right). Cilia projecting toward the posterior are denoted with yellow arrows, anterior projection with red arrows, and abnormal projection out of the plane of the figure with magenta arrows. Anterior is up.
Table 2.4.1. There is no difference in the mean length or mean range of motion between WT and MZvangl2 primary motile cilia.

<table>
<thead>
<tr>
<th>Cilia Type</th>
<th>Mean Length (µm) ± SD</th>
<th>Mean Range of Motion (°) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT KV</td>
<td>4.8 ± 0.8 n=82</td>
<td>50.0 ± 9.4 n=81</td>
</tr>
<tr>
<td>MZvangl2 KV</td>
<td>4.8 ± 0.8 n=82</td>
<td>51.4 ± 9.1 n=82</td>
</tr>
<tr>
<td>MZvangl2 + V1 MOa KV</td>
<td>4.5 ± 0.5 n=53</td>
<td>50.4 ± 7.8 n=53</td>
</tr>
<tr>
<td>MZvangl2 + V1 MOb KV</td>
<td>4.8 ± 0.6 n=31</td>
<td>50.1 ± 8.1 n=31</td>
</tr>
<tr>
<td>MZkneypek KV</td>
<td>5.0 ± 0.7 n=80</td>
<td>46.8 ± 8.8 n=80</td>
</tr>
<tr>
<td>vangl2 OE KV</td>
<td>4.6 ± 0.7 n=77</td>
<td>47.8 ± 8.3 n=77</td>
</tr>
<tr>
<td>WT neural tube</td>
<td>4.7 ± 0.9 n=49</td>
<td>48.7 ± 10.1 n=46</td>
</tr>
<tr>
<td>MZvangl2 neural tube</td>
<td>4.6 ± 0.7 n=34</td>
<td>45.7 ± 11.0 n=34</td>
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</tbody>
</table>
2.4.5 MZvangl2 mutants have defects in the asymmetric orientation and position of motile primary cilia in the ventral neural tube

To determine whether Vangl2 functions in orienting all motile cilia, or only those that are present in KV, I examined another population of primary motile cilia that line the ventral neural tube and floorplate. Arl13b-GFP labeling of these cilia showed that they also tilt posteriorly (arrows, Figure 2.4.6a,b) but the movement of all the motile cilia in this region obscured a more thorough analysis of asymmetries. To overcome this, I labelled donor embryos with Arl13b-GFP and transplanted a small population of these cells into unlabeled hosts. I found that in addition to the cilia having clear posterior tilting, they also project from the posterior end of the apical surface of these cells (Figure 2.4.6c). I confirmed the posterior positioning of cilia by labelling floorplate cells with the basal body marker, centrin-GFP. I found that 88% of basal bodies (n=73) were positioned at the posterior apical surface of floorplate cells (Figure 2.4.6d; Figure 2.4.7).

The establishment of planar polarity is believed to occur along the anterior-posterior axis of cells during neurulation, based on observations of neural cells expressing GFP-tagged Drosophila Prickle. Neural cells acquire apical-basal polarity at neural rod stages that occur between 12 and 18 somites [43]. Prickle is a regulator of PCP signaling and is initially localized along the entire anterior membrane of cells and becomes localized to the future apical surface as it develops along the neural midline (Figure 2.4.8a-c). By 20 somites, the cells have fully acquired AB polarity and the neural tube begins to lumenize at the midline. At this stage GFP-Prickle is clearly visible in pucti at the anterior apical surface of these cells and co-labelling with Arl13b-GFP showed at cilia are present and the opposite end of the apical surface (Figure 2.4.8c,d). The observed spatial arrangement of Prickle and cilia is consistent with a possible role for PCP signalling controlling the position of cilia outgrowth. I also wanted to assess whether the position of cilia is posteriorly biased, prior to the establishment of AB polarity. To study cilia positioning in isolated cells, I labelled WT cells with Arl13b-GFP and memb-mRFP, and transplanted labeled cells into unlabelled hosts. I then studied the position of cilia in relation to the anterior-posterior axis as the cells are acquiring AB polarity (12-18 somites). I found that cilia positioning is fairly random in neuroepithelial cells that are not yet polarized (12-14 somites), suggesting that the polarity of cilia positioning is not determined prior to the establishment of AB polarity (Figure 2.4.8e). I did however see a trend towards a posterior bias of cilia position as
neuroepithelial cells are developing AB polarity (16-18 somites), which may indicate that the posterior positioning of cilia is a characteristic of AB polarized cells (Figure 2.4.8e).

Having characterized the orientation and position of motile cilia in the ventral neural tube of WT embryos, I went on to determine whether this polarity was also controlled by PCP signalling. I examined the orientation of primary motile cilia in the ventral neural tube and floorplate of MZvangl2 mutant embryos by labelling them with Arl13b-GFP. I found that the floorplate was severely expanded in mutants, and that cilia lining the floorplate appeared misoriented (Figure 2.4.6e). However, it has previously been shown that MZvangl2 mutants have an ectopic accumulation of cells at the neural midline, and the overall abnormal environment where floorplate cilia are found, could be exerting a secondary affect on their orientation [43].

I next went on to determine whether PCP signalling is required for cilia positioning, I labelled MZvangl2 embryos with centrin-GFP and studied the positioning of basal bodies. I found that basal bodies were still properly docked at the apical surface but that their polarized localization was lost (Figure 2.4.6f,g; Figure 2.4.7). These results suggest that PCP signalling is required for the posterior orientation and positioning of primary motile cilia in the floorplate.
Figure 2.4.6. MZvangl2 mutants have defects in the asymmetric orientation and position of motile primary cilia in the ventral neural tube. (a-c) Confocal images of live WT embryos expressing the ciliary axoneme marker Arl13b-GFP (green) and membrane-localized mRFP (red). (a) Sagittal section through ~30 hpf neural tube, revealing motile posterior-tilted cilia lining floorplate (arrows). In this image, anterior is to the left. (b) Coronal section demonstrating posterior-oriented motile cilia lining the lateral floorplate (arrows) at ~30hpf. (c) Chimeric analysis of Arl13b-GFP expression revealing asymmetries in the position and orientation of floorplate cilia. (d) Centrin-GFP expression (green) demonstrating basal body docking at the posterior apical surface of WT floorplate cells. (e) Coronal section through the floorplate of ~30hpf MZvangl2 mutant embryos expressing memb-mRFP (red) and Arl13b-GFP (green). Defects in convergence and extension produce an expanded floorplate, and as a result, a greater number of ciliated cells are visible in MZvangl2 images (f,g) Coronal sections through the floorplate (f) and dorsal neural tube (g) of MZvangl2 embryos expressing Centrin-GFP (green) and memb-mRFP (red), demonstrating centralized localization of apically docked basal bodies. not, notochord; NT, neural tube; fp, floorplate. With the exception of panel (a), anterior is up.
Figure 2.4.7. PCP signalling controls the asymmetric localization of basal bodies to the posterior cell membrane. Quantification of the percentage of cells displaying an anterior, central, or posterior position of basal bodies at the apical surface of floorplate cells, in embryos that are wild-type (WT), MZvangl2 mutant, MZvangl2 mutants injected with 4ng of vangl1 morpholino (MZvangl2+v1MO), embryos injected with 300pg of wild type vangl2 mRNA (vangl2 OE), and MZknypek mutants. Results demonstrate that any disruption to PCP signalling disturbs the normal asymmetric localization of basal bodies to the posterior cell membrane. “a” indicates statistically significant difference from WT (p=<0.0001); “b” indicates statistically significant difference from all other samples (p=<0.0001).

Complete list of p values, as determined using a Chi-square test:

WT vs. MZvangl2: p=<0.0001; WT vs. MZvangl2+v1MO: p=<0.0001

WT vs. vangl2 OE: p=<0.0001; WT vs. MZknypek: p=<0.0001

MZvangl2 vs. MZknypek: p=<0.0001; MZvangl2+v1MO vs. MZknypek: p=<0.0001

vangl2 OE vs. MZknypek: p=<0.0001; MZvangl2 vs. MZvangl2+v1MO: p=0.0462

MZvangl2 vs. vangl2 OE: p=0.1465; MZvangl2+v1MO vs. vangl2 OE: p=0.0214
Figure 2.4.8. The spatial arrangement of Pk and cilia is consistent with a role for PCP signalling in controlling cilia positioning. (a-c) Coronal sections through the developing neural tube of WT embryos at the 8-somite (a), 14-16 somite (b) and 20-somite stage (c). Scatter-labelling of memb-mRFP (red) and GFP-Pk (green) expression was achieved through cell-transplantation into unlabelled host embryos at mid-blastula stages. GFP-Pk is asymmetrically localized to the anterior lateral membrane of neural progenitors at neural keel stages (arrows, a), shifts towards the neural midline as apico-basal polarity is established (b), and eventually marks the anterior apical surface of neuroepithelial cells (arrowhead, c) (d) The planar polarity of cilia formation is revealed as GFP-Pk (green) and Arl13b-mCherry (red) expression localize to opposite sides of the apical neuroepithelial cell membrane in 18-20 somite stage WT embryos. Polarity is best observed in isolated cells with large apical membranes (asterisks), and can be obscured in cell clusters that contain small apical processes from cells outside of the focal plane. (e) The anterior-posterior position of cilia on neuroepithelial cells at 12, 14, 16, and 18 somites. Cilia position was assessed through transplant experiments where WT cells were labeled with Arl13b-GFP and transplanted into unlabeled hosts.
2.4.6 Vangl2 has a cell autonomous function in controlling the posterior tilting of motile cilia in the ventral neural tube

MZvangl2 mutants have a severely disrupted neural tube, with an ectopic accumulation of cells at the midline, so it was unclear whether disrupted cilia orientation was a primary defect, or simply due to this abnormal environment [43]. To study cilia orientation in a normal environment and determine the cell-autonomous function of Vangl2, I performed cell transplants as described before. I labelled WT and MZvangl2 cells with Arl13b-GFP, transplanting them into WT unlabelled hosts and only studied labelled cells with motile cilia in the ventral neural tube. I determined the tilting of these cilia by measuring the angle of the ciliary axis relative to the posterior apical surface. WT cells displayed a distinct posterior tilting however in mutants, there was a shift to a more randomized distribution of angles and some cilia were even observed pointing in the anterior direction (WT n=49, MZvangl2 n=34, p=0.017; Figure 2.4.8e-g). These observations indicate that Vangl2 is also required for cilia tilting in the ventral neural tube and that this function is cell autonomous.

Additionally, I utilized these transplant experiments to measure the mean length and mean range of motion of WT and MZvangl2 mutant cilia in the ventral neural tube. As observed in KV, I found that cilia in MZvangl2 mutants (4.6 ± 0.9µm, n=34; 45.7 ± 11.0°, n=34) were of normal length and motion, when compared to WT cilia (4.7 ± 0.9µm, n=49; 48.7 ± 10.1°, n=46), (Table 2.4.1).
Figure 2.4.9. Chimeric analysis reveals a cell-autonomous function for Vangl2 in controlling the posterior tilting of motile floorplate cilia. (a, b) Examples of chimeric embryos, demonstrating Arl13b-GFP (green) expression in WT (a) or MZ\textit{vangl2} mutant (b) cells transplanted into the ventral neural tube of unlabelled WT host embryos. Anterior is up, as marked. (c) Graph of cilia orientation in WT vs. MZ\textit{vangl2} transplanted cells, as measured by the angle between the posterior apical cell surface and the central axis of ciliary motion. A statistically significant shift (p=0.017) in the MZ\textit{vangl2} ciliary axis is observed, as determined using a two-tailed student t-test.
2.4.7 MZvangl2 mutants still form multi-ciliated cells, but have misoriented primary motile cilia in the pronephric duct

Studies implicating PCP signalling in cilia formation have primarily focused on multi-ciliated epithelial cells in *Xenopus* [2, 3, 140]. These cells contain hundreds of motile cilia that generate unidirectional fluid flow across the tissue. Basal bodies rotate so that all cilia tilt in the same direction within a cell and this is coordinated between neighboring cells [11]. Our observations that Vangl2 is not required for the formation of motile primary cilia disagree with what was observed with downstream PCP effector molecules in multi-ciliated *Xenopus* cells [2, 3]. This could be explained by differences in the biogenesis of mono-ciliated and multi-ciliated cells.

I proceeded to examine whether cilia formation was disrupted on a population of multi-ciliated cells within the pronephric duct, a structure that gives rise to the zebrafish kidney tubule. The pronephric duct contains both mono and multi-ciliated cells that alternate along the length of the tubule and both have motile cilia, responsible for generating fluid flow [148]. Imaging of the pronephric duct in WT and MZvangl2 mutants revealed that the duct is severely expanded in mutants, likely due to disrupted CE movements that lengthen and narrow the tubule (Figure 2.4.10a,b). Cilia labeling using acetylated α-tubulin immunofluorescence revealed that multi-ciliated cells are still present in MZvangl2 mutant embryos. The cilia have a disorganized appearance when compared to those present on WT multi-ciliated cells, and this may be due to defects in their orientation (Figure 2.4.10a,b).

Since the WT and MZvangl2 mutant embryos I studied contained the Arl13b-GFP transgene, I co-labelled them with an antibody against GFP, which revealed that Arl13b-GFP only labels primary cilia and not multi-ciliated cells (Figure 2.4.10a,b). This was surprising considering the requirement for Arl13b in the formation of primary cilia. Arl13b regulates endocytic trafficking events that determine the composition of proteins within primary cilia, but different mechanisms may function in multi-ciliated cells [149].

To study the orientation of primary cilia in the pronephric duct, I performed *in vivo* imaging of Arl13b-GFP transgenic WT and MZvangl2 mutant embryos. Primary cilia in WT embryos were motile and had a defined posterior tilt, whereas imaging of MZvangl2 embryos confirmed that the duct is expanded and that the motile primary cilia are misoriented (Figure 2.4.10c,d). My results indicate that the formation of both primary cilia and those on multi-ciliated cells does not
require core PCP signalling. The role of Vangl2 in orienting cilia in the pronephric duct corresponds to the observations of motile cilia in KV and the ventral neural tube, suggesting a universal requirement of Vangl2 in orienting motile cilia.
Figure 2.4.10. MZvangl2 mutants display normal differentiation of multiciliated cells in the pronephric duct, but show defects in the orientation of primary motile cilia. (a,b) Sagittal confocal images through the pronephric duct of 48 hpf WT (a) and MZvangl2 mutant (b) embryos that express Arl13b-GFP. Anti-acetylated-tubulin staining (AcTub) is shown in red, and anti-GFP staining (Arl13b-GFP) is shown in green. Arrows indicate the presence of multi-ciliated cells (c,d) Live imaging of the PND of 48hpf WT (c) and MZvangl2 (d) embryos expressing Arl13b-GFP (green) demonstrates that the posterior-tilting of primary motile cilia is disrupted in MZvangl2 mutants. Anterior is to the left.
2.4.8 MZvangl2 mutants injected with vangl1 MO have normal cilia formation

My observations that MZvangl2 mutants have normal cilia length and motility are in contrast to the MO-mediated knockdown of Inturned, Fuzzy, Dvl, and Vangl2 in Xenopus embryos, which were all shown to cause defects in basal body docking and cilia formation [2, 3, 113]. Zebrafish have a paralog of Vangl2 know as Vangl1 that is 74% similar in amino acid sequence, but the two genes are expressed in mostly non-overlapping domains [153]. Sequence similarity suggests that there may be some functional redundancy, which raised the possibility that vangl1 expression may rescue cilia formation in MZvangl2 mutants. This is an unlikely scenario since vangl1 is not expressed until mid-somite stages and I have shown that ciliogenesis begins during gastrulation. Furthermore, vangl1 expression is not detected in the spinal cord so it is not likely to have any role in the formation of motile cilia in ventral neural tube or floorplate [153].

To definitively rule out the possibility that Vangl1 rescues cilia formation, I used an antisense MO (v1MOa) to block the translation of vangl1 RNA transcript in WT and MZvangl2 embryos. Injecting v1MOa did not produce a phenotype when injected into WT embryos, however equivalent concentrations of MO injected into MZvangl2 embryos produced more severe CE defects than in MZvangl2 embryos alone (Figure 2.4.11a-d). Analysis of cilia formation in v1MOa+ MZvangl2 embryos showed that cilia are still present and motile within KV and the floorplate (Figure 2.4.11e,f). While basal bodies are still docking at the apical surface of cells in v1MOa+ MZvangl2 embryos, their polarization is disrupted (Figure 2.4.11g,h; Figure 2.4.7).

Since there is no antibody against Vangl1, I verified my results using a second MO (v1MOb) to ensure vangl1 knockdown. The v1MOb targets the exon-intron boundary downstream of exon 5, and disrupts the splicing of vangl1 pre-mRNA (Figure 2.4.12a). I monitored the efficacy of v1MOb using RT-PCR and found that in embryos injected with 7ng of v1MOb, no WT vangl1 was detected. Instead, it resulted in the creation of two splice variants (one that includes introns 5-6, and one that results in an abnormal removal of exon 5) that both result in the formation of premature stop codon (Figure 2.4.12b). Analysis using v1MOb corroborated results obtained by injecting v1MOa, which revealed no defects in basal body docking and cilia formation, although cilia orientation was disorganized (Figure 2.4.12c-f). These findings exclude the possibility that Vangl1 rescues cilia formation in MZvangl2 mutants, further arguing that PCP signalling is not required for cilia formation.
Figure 2.4.11. Vangl1 does not rescue cilia formation in MZvangl2 mutants. (a-d) Lateral (top) and dorsal (bottom) views of 36 hpf MZvangl2 embryos that were injected with either 0ng (a), 2ng (b), 4ng (c), or 6ng (d) of a translation blocking vangl1 morpholino (v1MOa) at the one-cell stage. MZvangl2 normally display convergence and extension defects and exhibit axes that are shortened in the anterior-posterior direction, but expanded medio-laterally (a). Injection of v1MO results in a concentration dependent increase in convergence and extension defects and a more pronounced curling of the tail (b-d). (e-f) Confocal images of Arl13b-GFP (green) and memb-mRFP (red) expression in the 8-somite staged KV (e) and the 30hpf floorplate (f) of MZvangl2 embryos that have been injected with 4ng of v1MOa. Images demonstrate the formation of motile primary cilia (arrow, e) although the orientation of cilia appear disorganized (f). (g-h) centrin-GFP (green) and memb-mRFP (red) labeling in the floorplate (g) and dorsal neural tube (h) of 30hpf MZvangl2;v1MOa embryos reveal no defects in the docking of basal bodies to the apical cell membrane. However, asymmetric posterior localization of basal body docking and cilia outgrowth is lost (g).
Figure 2.4.12. Splice-site targeted vangl1 MO (v1MOb) efficiently blocks Vangl1 function. Genomic structure of vangl1 is shown (a), as is the binding site for v1MOb at the exon5/intron5-6 boundary, and the location of forward and reverse primers used to detect spliced vangl1 mRNA. Abnormal splicing events are also indicated. RT-PCR using for/rev1/rev2 PCR primers revealed the presence of two aberrantly spliced vangl1 messages after injection of v1MOb (b). All PCR amplicons were sequenced to identify the nature of each splice variant (data not shown). In lane i (uninjected control), only WT vangl1 cDNA is detected. In lanes ii and iii (embryos injected with 7ng and 14ng of v1MOb, respectively), no WT vangl1 is detected. Instead, the upper band (*), amplified by for/rev1 primer pairs, detects vangl1 message that includes intron5-6. The lower band (**), amplified by for/rev2 primer pair, detects an abnormal splicing event that removes vangl1 exon 5. Both splice variants result in the formation of premature termination codons, as verified by cDNA sequencing. (c-f) Arl13b-GFP expression in the 8-somite staged KV (c) and the 30hpf floorplate (d) of MZvangl2 embryos injected with 7ng of v1MOb demonstrate the formation motile primary cilia (arrows, c) although the orientation of cilia appear disorganized (d). (e-f) centrin-GFP (green) labeling in the floorplate (e) and dorsal neural tube (f) of 30hpf MZvangl2;v1MOb embryos reveal no defects in the docking of basal bodies to the apical cell membrane. However, asymmetric posterior localization of basal body docking and cilia outgrowth is lost (e).
2.4.9 Over-expression of \textit{vangl2} does not disrupt cilia formation

Vangl2 is considered a negative PCP regulator because it is required to restrict Fz/Dvl-induced cytoskeletal rearrangements to one side of a cell [17, 18]. To address the possibility that loss of Vangl2 is not sufficient to fully disrupt PCP, over-expression of \textit{vangl2} can be used to inhibit Fz/Dvl. I injected WT embryos with 300pg of WT \textit{vangl2} RNA and analyzed them at 24hpf. Embryos over-expressing \textit{vangl2} had a shorter body axis, curled-up tails, and were cyclopic, all of which are phenotypes associated with disrupted PCP signalling (Figure 2.4.13a,b). Importantly, these embryos displayed normal cilia formation, with defects in cilia orientation and position (Figure 2.4.13c-d, Figure 2.4.7). The fact that \textit{vangl2} over-expression phenocopies its loss of function is consistent with previous observations that with PCP signalling, loss and gain of function approaches often produce similar effects [154].
Figure 2.4.13. *vangl2* over-expression does not disrupt basal body docking and cilia formation. (a) Lateral view of a 36hpf WT embryo. (b) Lateral view of a 36hpf embryo that was injected with 300pg of *vangl2* mRNA at the one-cell stage. These *vangl2* overexpression (*vangl2OE*) embryos exhibit convergence and extension defects that are similar to the MZ*vangl2* phenotype. (c,d) Confocal images through the 8-somite staged KV (d) and the 30hpf floorplate (e) of *vangl2OE* embryos expressing Arl13b-GFP (green) and memb-mRFP (red). Images demonstrate the normal formation motile primary cilia (arrow, c) although the orientation of cilia appear disorganized (d). (e) Centrin-GFP expression (green) in *vangl2OE* embryos demonstrates centralized basal body position on floorplate cells, but apical docking is not affected (arrowhead).
2.4.10 MZknypek mutants have similar defects in the posterior orientation and position of primary motile cilia

To determine whether the function of Vangl2 in orienting and positioning cilia is shared with other PCP factors, I focused on studying the *knypek* mutant. Knypek is the zebrafish ortholog of Glypican 4 and is a member of the heparan sulfate proteoglycan family [50]. It is believed the physically bind Wnt ligands and function as a positive regulator of PCP signalling [50].

MZknypek mutants were generated using a germline replacement strategy (Simone Superina) and were observed to have more severe CE defects when compared to zygotic *knypek* mutants (Figure 2.4.14a,b). Arl13b-GFP labelling revealed that cilia were still present and motile in MZknypek embryos (Figure 2.4.14c). I observed misoriented cilia in the pronephric duct and on the floorplate, demonstrating a requirement for Knypek in orienting motile cilia (Figure 2.4.14d,e). Analysis of basal body position using centrin-GFP showed that basal bodies were still docked at the apical surface but their posterior localization was disrupted at the floorplate, albeit to a lesser extent than what was observed in MZvangl2 embryos (Figure 2.4.14f,g; Figure 2.4.7). Analysis of MZknypek embryos supports the idea that PCP signalling is not required for cilia formation, but is required for the positioning and tilting of motile cilia.
Figure 2.4.14. Analysis of MZknypek mutant embryos reveals similar defects in the asymmetric localization and orientation of primary motile cilia. (a-b) Lateral (top) and dorsal (bottom) views of knypek (a) and MZknypek (b) mutant embryos at 24 hpf. MZknypek mutant embryos are more severely affected than zygotic knypek counterparts, displaying a broader medial-lateral axis (arrows in a and b), greater compression of somites, and a more severe yolk-extension phenotype (asterisks in a and b). (c-e) Live imaging of Arl13b-GFP expression in the 8-somite stage KV (c), 48hpf PND (d), and ~30hpf floorplate (e) of MZknypek mutant embryos demonstrating the formation of motile primary cilia. Cilia lining the floorplate and PND of MZknypy have lost normal posterior orientation (arrowhead, d). (f-g) Live imaging of cen-GFP expression at the floorplate (f) and dorsal neural tube (g) of MZknyp mutant embryos demonstrating normal docking of basal bodies to the apical membrane. Asymmetric localization of basal bodies and cilia outgrowth to the posterior membrane is disrupted (f). *ant, anterior direction is as indicated.*
2.5 Discussion

To our knowledge, Arl13b-GFP is the first reported marker that labels cilia in living embryos and provides an unprecedented ability to study cilia dynamics. Arl13b-GFP specifically labels cilia along the entire length of the axoneme and has obvious advantages over conventional α-acetylated tubulin immunofluorescence techniques. For one thing, it enables the study of cilia movement using conventional laser confocal microscopy, providing information like the range of motion of cilia. Arl13b-GFP can also be used to more precisely study cilia orientation and position, which are often not possible with immunofluorescence since it disrupts tissue architecture.

I used this tool to study cilia formation in MZvangl2 mutants and determined that primary motile cilia are still present and have normal motion. However, I have implicated PCP signalling in controlling the posterior orientation and positioning of these cilia in KV, the ventral neural tube, and the pronephric duct. Indeed the asymmetric distribution of GFP-Pk and cilia in polarized neuroepithelial cells is consistent with a role for PCP in controlling the position of basal body docking. I have also shown that cilia positioning in neuroepithelial cells is random prior to the establishment of AB polarity, suggesting that PCP signalling controls the position of cilia only in already polarized cells. I have also shown that cilia positioning is random prior to the establishment of AB polarity in neuroepithelial cells. In polarized cells, I have shown that PCP signalling regulates the positioning of primary motile cilia, but it is still unclear whether it also regulates the asymmetric positioning of immotile cilia. Analysis of MZknypek mutants showed that they too have defects in the posterior tilting and positioning of motile primary cilia, but that cilia formation is otherwise normal. Our findings were supported by a subsequent study by Song et al. (2010), where they generated a mouse mutant where both vangl1 and vangl2 were eliminated. Double vangl1/vangl2 mutants showed defects in left-right asymmetry resulting from turbulent nodal flow. Analysis of cilia formation revealed normal basal body docking, cilia outgrowth, and cilia motility, but they observed a randomization in ciliary positioning [115].

Taken together, analysis of MZvangl2 zebrafish mutants and vangl1/vangl2 mouse mutants indicates that Vangl2, and thus core PCP signalling, is not required for cilia formation. This suggests that the proposed functions of Dvl, and PCP effectors like Inturned and Fuzzy in basal body docking and cilia formation, are likely independent of their roles in PCP signalling [2, 3].
Furthermore, recent studies examining *inturned* and *fuzzy* mouse mutants have also raised doubts as to whether their functions in cilia formation are related to PCP signalling. These mutants have defects in basal body docking and cilia formation, but do not exhibit any CE defects [109, 110]. Since CE defects are a hallmark of disrupted PCP in vertebrates, it suggests that Inturned and Fuzzy do not act as “PCP effectors” in the mouse.

There are still other aspects of the relationship between PCP signalling and cilia formation that remain unclear. Mouse mutants that lack Celsr2 and Celsr3, orthologs of *Drosophila* Flamingo, have defects in basal body docking and cilia formation in multi-ciliated ependymal cells [111]. Celsr proteins are cadherins that maintain the asymmetric distribution of Fz and Vang proteins across membranes, and are typically considered core PCP factors [15]. These findings suggest that either Celsr proteins have uncharacterized PCP-independent functions, or that core PCP factors behave differently with regards to cilia formation. Interestingly, Primary cilia that are present in the choroid plexus were normal, suggesting that Celsr2/3 function in cilia formation only in specialized multi-ciliated cells [111].

Even studies examining the function of Vang proteins show discrepancies. Antic et al. (2010) reported that *vangl1* mutant mice have a weakly penetrant defect in left-right asymmetry due to disruptions in the planar polarization of node cilia. The same study reported using a MO to knockdown *vangl2* function in *Xenopus* embryos. They found that the assembly of cilia in the gastrocoel roof plate (GRP), a structure analogous to KV and the node, was normal but that their posterior localization was lost [58]. This was perplexing since Mitchell et al. (2009) reported using a *vangl2* MO also in *Xenopus* embryos and observed defects in cilia formation in multi-ciliated epithelial cells [113]. Moreover, shortly after our work was published, May-Simera et al. (2010) reported studying a mutant of *vangl2* in zebrafish and they observed fewer and shorter cilia within KV, resulting from defects in basal body docking [116]. Their results differ from ours, but so does the mutant allele that they studied. The allele we used was *tk50f* which is a deletion of the open reading frame and is believed to be a null because no transcript is detected. They used the *m109* allele that causes a premature termination in the last exon so it is possible that it may display neomorphic activity and may not represent the loss of function phenotype (refer to section 4.2) [27].
In summary, based on our studies of Vangl2 and Knypek, we conclude that PCP signalling is not required for cilia formation. I have however implicated PCP signalling in controlling the posterior tilting and positioning of motile primary cilia. Analysis of other mutants of core PCP factors will be instrumental clearing up some of the debate in the field as to the role of core PCP signalling.
Chapter 3

3. Investigating the role that cilia play in establishing planar cell polarity
3.1 Abstract

Cilia function as cell antenna for the perception of diverse environmental cues including factors for the Hh and PDGF developmental signalling pathways. Recent evidence also suggests that cilia may be involved in regulating PCP. Cilia became implicated in PCP signalling when CE defects were observed with the knockdown of certain proteins (Inversin, BBS proteins, Ofd1, Lrrc6l) that localize at or near cilia and basal bodies [4-8]. Curiously, zebrafish mutants affecting IFT do not show CE defects, despite the fact that IFT proteins have recognized roles in cilia formation [131]. The observation that cilia persist till 4dpf in zebrafish IFT88 mutants suggested that initial cilia formation may be rescued by maternally supplied WT RNA and protein. I utilized a germ-line replacement (GLR) strategy to generate complete loss of function mutants of IFT88 (MZift88), where both maternal and zygotic gene products are eliminated. Analysis of MZift88 mutants revealed that cilia formation is almost completely abolished, permitting the analysis of the early functions of cilia in zebrafish development. I proceeded to do an in depth analysis of PCP and found that CE movements and the polarization of basal bodies were normal, suggesting that cilia are not required for regulating PCP. Interestingly, I found that MZift88 mutants had defects in OCD at gastrulation and neurulation and that this function of IFT88 was independent of its role in cilia formation. Since OCDs play an important role in vertebrate development, implicating IFT88 in controlling these processes in a cilia-independent manner may have important consequences for the interpretation of cilia function in human disease.
3.2 Brief Introduction and Rationale

Cilia first became implicated in modulating aspects of Wnt signalling from studies that analyzed the function of Inversin. Inversin localized to cilia, and its knockdown resulted in cystic kidneys and left-right patterning defects, both of which are typically associated with cilia dysfunction [121, 122]. Interestingly, while nodal flow was disrupted, cilia in *inversin* mutant mice seemed otherwise normal [122]. Simons et al. (2005) showed that these mutants also had defects normally associated with PCP signalling, like disrupted hair patterns, in addition to their ciliary defects. Furthermore, knockdown experiments in *Xenopus* embryos showed that Inversin was required for PCP-mediated CE movements. Inversin was shown to inhibit canonical Wnt signalling by targeting cytoplasmic Dvl for degradation, but it promotes PCP signalling by permitting Dvl to accumulate at the plasma membrane [8]. Subsequent studies that examined the functions of other cilia-associated proteins (BBS1, 4, 6; Ofd1; Lrrc6l) further supported the notion that cilia regulate PCP-mediated CE movements [4-7]. Based on these collective observations, a model was proposed whereby the bending of primary cilia is required to elicit an intracellular response that restricts canonical Wnt signalling, and in turn promotes PCP signalling. When cilia are disrupted, the balance shifts in favour of canonical Wnt signalling and PCP-associated defects are observed [79].

A parallel branch of research is suggesting that cilia may play a more indirect role in regulating PCP. Studies of multi-ciliated *Xenopus* epithelial cells and mouse brain ependymal cells found that cilia-generated fluid flow regulated the PCP of these same cells. Specifically, cilia-generated fluid flow was required to orient basal bodies, and thus cilia, in a uniform direction [140, 141]. This function of cilia is supported by the PCP-related defects that accompany the defects in ciliary flow that are observed in *inversin* mutant mice, despite otherwise normal cilia [122]. However, whether cilia-generated fluid flow plays any role in regulating the PCP of monociliated cells remains unstudied.

While the evidence that cilia play a role in PCP is persuasive, key issues remained. One issue was that the ciliary proteins implicated in regulating CE movements do not function exclusively within cilia. Therefore, their role in regulating CE movements is not necessarily due to their ciliary localization/function. Indeed, since most vertebrate cells form a primary cilium, distinguishing between cilia-dependent and independent functions poses a challenge. Also, many
of the CE defects observed in zebrafish were obtained using MO-mediated knockdown, which is known to cause off-target affects, emphasizing the importance of examining cilia-specific mutants [4-6].

One of the most extensively characterized cilia proteins is IFT88, a component of the IFT machinery required for anterograde axonemal transport. In zebrafish, the ift88 mutant is called polaris, and is reported to have cystic kidneys, laterality defects, hydrocephalus, the eventual loss of all sensory cilia, and ultimately lethality by 5 dpf. However, defects in PCP-mediated morphogenic movements were not observed [131]. Interestingly, initial ciliogenesis does not appear to be affected in these mutants, suggesting that IFT is required for cilia maintenance but not formation. However, in mouse ift88 mutants, initial ciliogenesis is disrupted and this leads to neural tube closure defects, mis-oriented cochlear stereocilia, and an enlarged pericardial sac, in addition to the laterality and kidney defects seen in zebrafish. The neural tube and stereocilia defects were attributed to defective Hh and PCP signalling, respectively [129].

The absence of PCP-related defects in zebrafish ift88 mutants may be due to the fact that cilia still persist during early development. Furthermore, I hypothesize that maternal IFT88 gene products (from heterozygote mothers) may enable cilia to form in zygotic ift88 mutant embryos, but when maternal stores are exhausted, the cilia are not maintained. My objective was to eliminate maternal contribution and generate MZift88 mutants in order to determine how the complete loss of cilia affects PCP signal transduction.

Here, I report that MZift88 mutants lack all cilia formation yet PCP signalling is normal. I implicate IFT88 in controlling both PCP-dependent OCD during gastrulation and PCP-independent OCD during neurulation, and show that this function of IFT88 is cilia-independent.
3.3 Materials and Methods

3.3.1 Zebrafish strains

Established zebrafish husbandry protocols were adhered to for all strains used throughout the duration of the study and all protocols were approved by the Animal Care Committee at the Hospital for Sick Children. The ovlf\textsuperscript{288b} (ift88) mutant allele was recovered in a chemically induced mutagenesis screen. Mutants have a T to A base pair transition that leads to a premature stop codon in exon 12 [131, 155]. MZcep290 mutant embryos were a gift from the lab of Cecilia Moens (Fred Hutchinson Cancer Research Center) and were generated from the mating of zygotic cep290 mutants. The cep290\textsuperscript{fh297/Q1210*} mutant allele was isolated in a TILLING mutant screen and contains a nonsense mutation that truncates halfway through the protein (TILLING Database).

3.3.2 Germ-line replacement strategy

A previously published germ-line replacement strategy was used to obtain MZift88 mutant embryos [143]. In brief, ift88 +/- zebrafish were intercrossed and their progeny (host embryos) were injected with GFP-nanos 3’UTR RNA to specifically label their germ cells. Concurrently, WT donor embryos were injected with a dead-end MO (Gene Tools, LCC) to specifically ablate their germ cells. At mid-blastula stages (4hpf), approximately 50-100 cells were transplanted from the margin of host cells into the animal cap of donor embryos, with the goal of transplanting mutant germ cells into WT donor embryos. The objective is to create germ-line chimeras that are somatically WT but have an ift88-/- germ-line. Adult germ-line chimeras were crossed to known ift88-/- fish and their progeny were screened. Female germ-line chimeras that are positive for an ift88 +/- germ-line will have 50% MZift88 embryos in their progeny, which are identified by their curved body axis.

3.3.3 Embryo microinjections and in vivo localization

Plasmids encoding the open reading frames of desired genes were linearized and the mMESSAGE mMACHINE system (Ambion) was used for the in vitro synthesis of mRNA. The following concentrations of mRNA were injected into one-cell stage embryos for in vivo localization studies: 20pg of membrane-localized monomeric red fluorescent protein (memb-
mRFP), 2.5pg of GFP-tagged *Xenopus* Centrin (Cen-GFP; Adrian Salic, Harvard University, Boston), 7pg of Arl13b-GFP, and 100pg of Histone 2A-mCherry. Embryos at the relevant stages were mounted on a coverslip in 0.8% agarose and imaged using a Zeiss 710 laser scanning confocal microscope.

**3.3.4 Transmission electron microscopy (TEM)**

TEM was performed at the Advanced Bioimaging Center (SickKids) to generate serial sections through the neural tube and image cilia in MZift88 mutants at 30hpf. Dechorinated embryos were fixed with 2% glutaraldehyde in 0.1M Sorenson’s Phosphate buffer (pH 7.4) at 4°C overnight. Samples were washed in 0.1M cacodylate buffer (pH 7.3) and post-fixed in 1% osmium tetroxide + 1.25% potassium ferrocyanide in 0.1M cacodylate buffer for 1.5 hrs. Samples were stained with 4% aqueous uranyl acetate for 1hr and dehydrated through ethanol series. Samples were infiltrated with Epon and propylene oxide (0:1, for 30 min; 1:1, for 2hrs; 3:1, for 3hrs; 1:0, overnight). Samples were placed in silicone molds filled with Epon and polymerized in a 70°C oven. Serial sections were cut on a Leica Ultracut UCT ultramicrotome and stained with lead citrate and uranyl acetate. Sections were viewed with an FEI Tecnai F20 transmission electron microscope and images were captured with an AMT 16000 digital camera.

**3.3.5 Whole mount RNA *in situ* hybridization**

To study CE movements occurring during somitogenesis, axis extension was assessed at the 10-somite stage (14 hpf) using *krox20* (rhombomeres 3 and 5) and *myoD* (somites and adaxial cells) riboprobes. CE of the embryonic axis was studied at bud-stage (10hpf) using *ntl* (notochord), *hgg1* (prechordal plate), and *dlx3* (anterior edge of the neural plate) riboprobes. Riboprobes were generated from linearized DNA plasmids by *in vitro* transcription using a DIG RNA Labelling Kit (Roche), digoxigenin-11-UTP (Roche), and T3 polymerase (Roche). Standard protocols for whole-mount *in situ* hybridization of zebrafish embryos were used. Embryos were subsequently cleared in 100% methanol and mounted in benzylbenzoate/benzylalcohol (2:1) prior to imaging on an Axio Imager.M1 compound microscope (Zeiss).
3.3.6 Cell transplantation

Cell transplants were utilized to study cilia orientation in the absence of cilia-driven fluid flow. One-cell stage WT embryos were injected with Arl13b-GFP and mRFP to label cilia and plasma membranes, respectively. Transplants of approximately 50-100 labeled WT donor cells into MZift88 mutant hosts were performed using established techniques at mid-blastula stages (4hpf). Embryos were mounted on a coverslip in 0.8% agarose and imaged using a Zeiss 710 laser scanning confocal microscope. Only labeled cells that formed motile cilia and were transplanted into the ventral neural tube were analyzed and z-stack projections were generated.

3.3.7 Oriented cell division analysis

To analyze OCD occurring during gastrulation, I labeled WT, Mift88 mutant, and MZift88 mutant embryos with Arl13b-GFP (cilia and slight membrane labeling) and histone 2B-Cherry (histone labeling) by injecting RNA into one-cell stage embryos. Histone2B-Cherry labeling enables the identification of dividing cells, while Arl13b-GFP membrane labeling enables the measurement of the division angle. Arl13b-GFP cilia labeling is also used to distinguish between Mift88 (that form cilia) from MZift88 mutant embryos (that do not form cilia) at stages when cilia formation has begun (after 9hpf). At shield stage (6hpf), I mounted embryos in agarose with the dorsal embryonic shield facing the coverslip and took confocal 2µm z-series over a 2hr time period (every 3min). I subsequently measured the angle of cell division in relation to the embryonic margin (denoted as 0°) so that cell divisions that occur perfectly in the animal-vegetal axis have a division angle of 90°. To examine OCD occurring during neurulation, WT and MZift88 mutant embryos were labeled with Arl13b-GFP and histone 2B-Cherry as described above. At 6-somites (12hpf), I mounted embryos in agarose with the neural keel facing the coverslip and took confocal 1µm z-series over a 1hr time period (every 3min). These neuroepithelial cells undergo a rotation in order to divide across the neural midline. I first measured the initial position of the metaphase plate in relation to the neural midline (denoted 0°) and then measured the final position of the metaphase plate after the cells undergo rotation.
3.3.8 Immunofluorescence

Microtubule staining in zebrafish was performed according to Solnica-Krezel and Driever (1994), with minor modifications[156]. In brief, dechorinated embryos were fixed with 3.7% formaldehyde + 0.25% glutaraldehyde + 2% TritonX-100 in microtubule assembly buffer (80mM K-PIPES pH 6.5 + 5mM EGTA + 1mM MgCl$_2$ in water) for 2-3hrs on a rocker. Embryos were incubated for post-fixation overnight at -20°C in 100% methanol and rehydrated the next day with a methanol series. Embryos were blocked for 1hr at room temperature with 2% BSA + 2.5% goat serum in TBS and incubated with an anti-mouse α-tubulin antibody (1:500, Sigma) in 2% BSA + 5% goat serum in TBS. This was followed by incubation with goat anti-mouse Alexa 488 secondary antibody (1:1000, Invitrogen) in 2% BSA + 5% goat serum in TBS. Embryos were cleared in glycerol, mounted in 0.8% agarose, and imaged using a Zeiss 710 laser scanning confocal microscope.

3.3.9 Statistical analysis

Measurements to assess CE movements including the anterior-posterior extent of krox20 expression, medio-lateral extent of myoD expression, the gap between the prechordal plate and notochord, and body axis extension angle were all compared using a 2-tailed student T-test and statistical significance was assigned when the P-value was less than 0.05 (alpha). Angles measuring the orientation of cell division during gastrulation and neurulation were compared using a Watson-Williams two-sample test for circular data and statistical significance was assigned when the P-value was less than 0.05 (alpha).
3.4 Results

3.4.1 MZift88 mutants have defects in cilia formation

In an attempt to eliminate cilia formation in early zebrafish development, I sought to generate MZift88 embryos, where both the maternal and zygotic ift88 gene product has been completely eliminated. To do this, I employed a germ-line replacement strategy to generate germ-line chimeras that are somatically WT but harbor an ift88/- germ-line [143]. I was able to successfully generate female ift88/- chimeras, but as expected, male ift88 +/- chimeras were sterile, likely due to sperm immotility [88]. Thus, in order to obtain MZift88 mutant embryos, I mated female ift88/- germ line chimeras to ift88+/-- males to generate progeny where 50% would be maternal mutants (Mift88) and 50% would be MZift88 mutants. Mift88 and MZift88 embryos are developmentally delayed but by 30hpf, Mift88 mutants resemble WT embryos while MZift88 mutants resemble their zygotic counterparts and develop a curved body axis (Figure 3.4.1a,b). However, MZift88 mutants do not develop any other obvious defects in patterning and morphogenesis that would indicate significant defects in PCP signalling, like a shorter and broader body axis (Figure 3.4.1a,b) [25].

To determine whether ciliogenesis was eliminated in MZift88 mutants, I injected one-cell stage embryos with Arl13b-GFP RNA, an in vivo marker of the ciliary axoneme, and analyzed cilia formation at 9hpf. As I have previously shown, cilia formation begins during late gastrulation at approximately 75-80 % epiboly (8hpf, see Chapter 2). By 9hpf short cilia are clearly visible in WT embryos (asterisk; Figure 3.4.1c) and these cilia are absent in MZift88 embryos (Figure 3.4.1d). However, I observed the persistence of small green puncti at the membrane of some cells, which I suspected to be remnants of the ciliary membrane (asterisk; Figure 3.4.1d). To determine how much of the cilium remains in MZift88 mutants, I performed TEM on 30hpf embryos. I found that basal bodies remained properly docked at the apical membrane, but with the exception of a small membrane patches, the ciliary axoneme is completely absent (Figure 3.4.1e). Analysis of MZift88 mutants indicates that the persistence of cilia in zygotic ift88 mutants is due to maternal rescue, and that IFT is in fact required for cilia formation in zebrafish.
Figure 3.4.1 MZift88 mutants have defects in cilia formation. (a) Lateral view of a 3 day-old WT embryo. (b) Lateral view of a 3 day-old MZift88 embryo exhibiting a curved body axis, pericardial edema (arrowhead), hydrocephalus (asterisk), and a cystic kidney (arrow). (c-d) Confocal images of WT (c) and MZift88 mutant (d) embryos expressing Arl13b-GFP (green) and memb-mRFP (red) at 9hpf. Lateral views of the mesodermal germ layer during late gastrulation reveals short cilia in WT embryos (asterisks; c) but only small Arl13b-GFP puncti in MZift88 mutants (asterisks; d). (e) Transmission electron microscopy micrograph of a cilium in the neural tube of a 30hpf MZift88 mutant embryo reveals defects in axoneme elongation but normal apical docking of basal bodies.
3.4.2 The ciliary axoneme is not required for PCP-mediated CE movements

The most obvious outcome of disrupted PCP signalling in vertebrates is a defect in CE movements that are required to lengthen and narrow the body axis [25]. Examining the morphology of MZift88 mutants did not reveal any obvious CE defects but they could be subtle. To conclusively determine whether cilia were required for PCP signalling, I proceeded to analyze whether PCP-mediated CE movements were disrupted in MZift88 mutants using three different approaches. To obtain MZift88 embryos, I mated an ift88 +/- germ-line chimera female to an ift88 +/- male and analyzed the resulting progeny (50% Mift88, 50% MZift88). First, I looked at the extension of the body axis using RNA in situ hybridization with the markers krox20 (rhombomeres 3 and 5) and myoD (somites and adaxial cells) at 10 somites (14hpf) [29, 30]. Defects in PCP signalling typically lead to a compressed body axis and a wider hindbrain and somites, but a comparison of Mift88/MZift88 embryos to WT counterparts revealed no statistical differences in the width of krox20 (WT n=21; Mift88/MZift88 n=21) and the length of myoD (WT n=21; Mift88/MZift88 n=20) expression (Figure 3.4.2a-c).

Body axis extension can also be assessed in living embryos that are undergoing somitogenesis. As the body axis extends over the yolk, the distance between the head and the tail becomes narrower. Body axis extension can be represented as an angle with its vertex at the center of the yolk where the two rays measure the head and the tail, and the vertex meets at the center of the yolk. When PCP signalling is disrupted, the body axis does not properly extend over the yolk, resulting in a greater distance between the head and the tail, and an expanded body axis extension angle [5]. A comparison of 8-somite WT (n=23) and Mift88/MZift88 (n=20) embryos also revealed that the body axis extension angle was not statistically different (Figure 3.4.2e-g).

Finally, I analyzed at the migration of the prechordal plate, one of the earliest readouts of PCP-mediated CE movements occurring during gastrulation [44]. This can be visualized with RNA in situ hybridization using the markers ntl (notochord), hgg1 (prechordal plate), and dlx3 (anterior edge of the neural plate) at bud-stage (10hpf). When CE movements occur normally, the migration of the prechordal plate aligns with the border of the neural plate, but when CE movements are disrupted, these structures are misaligned [44]. This migration can be quantified by measuring the gap between the prechordal plate and the notochord and a comparison of WT
(n=21) and Mift88/MZift88 (n=23) mutants found that this gap was not statistically different, indicating that early CE movements are unaffected (Figure 3.4.2h-j).

Overall, based on a combination of different approaches, I concluded that PCP-mediated CE movements are normal in MZift88 mutants. Since cilia are almost completely disrupted in MZift88 mutants, it suggested that the ciliary axoneme is not required for PCP signal transduction.
Figure 3.4.2 Cilia are not required for PCP-mediated CE movements. (a-d) Whole-mount flat-mount RNA *in situ* hybridization showing representative 10-somite stage WT (a) and Mift88/MZift88 (b) embryos with krox20 and myoD riboprobes. Quantification of the medio-lateral extent of krox20 (c; p=0.06) and the anterior posterior extent of myoD expression (d; p=0.21) demonstrate no significant differences in CE of the body axis between WT (n=21) and Mift88/MZift88 mutants (n=21, krox20; n=20, myoD). (e-g) DIC images of representative WT (c) and Mift88/MZift88 (d) embryos taken at approximately 8-somites and imaged from the lateral side to visualize the body axis extension angle. Quantification of the body axis extension angle (g) in WT (n=23) and Mift88/MZift88 mutants (n=20) demonstrated no significant differences (p=0.36). (h-j) Whole-mount RNA *in situ* hybridization showing representative bud-stage WT (e; n=21) and Mift88/MZift88 (f; n=23) embryos with ntl, hgg1, and dlx riboprobes. Ntl, hgg1, dlx expression demonstrate normal migration of the prechordal plate in Mift88/MZift88 and quantification of the gap between the prechordal plate and the notochord (j) revealed no significant differences (p=0.10). Statistical significance for all experiments was determined using a 2-tailed student T-test and alpha was set as 0.05.
3.4.3 Cilia-generated fluid flow is not required for basal body positioning but may be required for their orientation

Studies of multi-ciliated epithelial cells suggested that cilia participate in a positive feedback loop where cilia-generated fluid flow regulates the polarity of these same cilia [140, 141]. The absence of a ciliary axoneme in MZift88 embryos prevents the generation of any cilia-driven fluid flow and I wanted to exploit this phenotype to determine what role, if any, cilia-driven fluid flow had on the positioning and orientation of basal bodies/cilia. To determine whether cilia-generated fluid flow is required for cilia positioning, I labeled the membranes (memb-mRFP) and basal bodies (Cen-GFP) of WT and MZift88 mutant embryos and studied basal body position on the apical surface of floorplate cells at 28-32hpf. At this stage, MZift88 mutant embryos can be distinguished from Mift88 embryos by their curved body axis and a neural tube that does not lumenize to the same extent. As I reported in Chapter 2, I found that a majority of basal bodies in WT cells (85%; n=27) favor a position at the posterior end of the apical surface (Figure 3.4.3a,c). MZift88 mutant cells also show this posterior bias (88%; n=27), suggesting that cilia-generated fluid flow is not required for basal bodies/cilia positioning (Figure 3.4.3b,c).

Next, to determine whether cilia orientation is disrupted in the absence of flow, I performed transplant experiments. I labeled the cilia of WT cells using Arl13b-GFP and transplanted them into unlabeled MZift88 hosts to assess how WT cilia behave in an environment where there is no cilia-generated fluid flow. I focused on motile cilia on cells transplanted into the ventral neural tube, since I have previously shown that these cilia have a defined posterior orientation (Chapter 2). Most transplants did not yield motile cilia, even when cells were transplanted into the correct region of the neural tube, and this may be due to the defects in neural tube lumenization in MZift88 mutants. Of the transplanted cells that did form motile cilia, they exhibited a normal posterior orientation. However, I have some preliminary evidence that there may be a defect in the reinforcement of cilia orientation. Analysis of z-stack projections through a ciliated cell showed a cilium that swayed, where it was initially oriented posteriorly, then quickly switched to an anterior orientation, before reverting back to a posterior orientation (Figure 3.4.4a-c). Previous experiments (Chapter 2) where I transplanted WT Arl13b-GFP labeled cells into an unlabeled WT background (n=49) never yielded cilia with any visible swaying (Figure 2.4.9). This suggests that cilia-generated fluid flow may be required for the orientation of motile cilia, by reinforcing the orientation that is initially established by PCP signalling.
Figure 3.4.3 Cilia-generated fluid flow is not required for the posterior positioning of basal bodies on floorplate cells. (a-b) Coronal section through the floorplate of 28-32hpf WT (a) and MZift88 mutant (b) embryos expressing memb-mRFP (red) and Centrin-GFP (green) demonstrating basal body docking at the posterior apical surface of in both WT (a; yellow arrows) and MZift88 mutant cells (b; yellow arrows). (c) Quantification of the percentage of either WT (n=27) or MZift88 (n=27) cells displaying anterior (blue), central (red), and posterior (purple) position of basal bodies at the apical surface of floorplate cells.
Figure 3.4.4 Cilia-generated fluid flow may be required to reinforce the posterior orientation of motile cilia in the ventral neural tube. (a-c) Chimeric embryo with WT cells expressing Arl13b-GFP (green) and memb-mRFP (red) transplanted into the ventral neural tube of unlabeled MZift88 host embryos. Z-stack projections showing a cilium that is initially oriented posteriorly (a) but then switches to an anterior orientation (b) before switching back to a posterior orientation (c).
3.4.4 IFT88 plays a cilia-independent role in controlling OCD during gastrulation

In addition to controlling CE movements, PCP signalling has also been implicated in controlling specific OCD. During gastrulation, cells of dorsal tissues have been shown to divide along the animal-vegetal (AV)/anterior-posterior (AP) axis. This process requires PCP signalling and was suggested to contribute to axis elongation [36]. To determine whether this aspect of PCP signalling function is affected in MZift88 mutants, I monitored dorsal cell divisions of shield-stage (6hpf) embryos over time (Figure 3.4.5a). To do this, I labelled WT, Mift88 mutant, and MZift88 mutant embryos with Arl13b-GFP (cilia and slight membrane labelling) and histone 2B-Cherry (labels histones) to monitor the orientation of cell division in relation to the embryonic margin, which was denoted as 0° (Figure 3.4.5a-c). I took confocal z-series over a two-hour time period starting from shield-stage (6hpf) to 75% epiboly (8hpf). Consistent with published observations, I found that WT cell divisions (n=120) favor a 90° angle from the margin and are thus aligned with the AV/AP axis (Figure 3.4.5b,d) [36]. In contrast, divisions in Mift88 mutants (n=161) show a statistically significant shift to a more randomized division angle profile and in MZift88 mutants (n=78) this profile is even further randomized (Figure 3.4.5c,d).

At first glance, our results may suggest a role for cilia in orienting polarized cell divisions. Indeed, it has been proposed that sensory inputs to the cilium prior to cell division may bias the ultimate direction of division, even though cilia are disassembled before a cell divides [157]. However in our system, I have shown that cilia only first start forming at approximately 75-80% epiboly, and that the OCD analyzed during early gastrulation stages (shield-75% epiboly) actually occur before cilia are normally present (Figure 3.4.5e,f). Early zebrafish development thus provides an elegant model system for investigating cilia-independent protein function. Based on my analysis of MZift88 mutants, I must therefore conclude that IFT88 plays a cilia-independent function in controlling OCD occurring during gastrulation.
Figure 3.4.5 IFT88 plays a cilia-independent role in controlling OCD during gastrulation. (a) Schematic diagram representing a shield-stage (6hpf) embryo showing the dorsal region (red square) that was imaged using confocal z-series over a 2h time period. The orientation of cell division is determined by measuring the angle between the embryonic margin (set as 0°) and the line along which the cell is dividing. Divisions that are perfectly aligned with the animal-vegetal axis will have a division angle of 90°. (b-c) Confocal images of 6-8hpf WT (b) and MZift88 mutant (c) embryos expressing Arl13b-GFP (green; cell membranes) and histone 2B-Cherry (red; histones) used to visualize cell divisions. WT cell divisions typically divide at an angle 90° from the embryonic margin (yellow arrows, b) while cell divisions in MZift88 mutants are randomized, with some occurring perpendicular to WT divisions (yellow arrows, c). (d) Graph of the orientation of cell divisions as measured by the percentage of divisions that deviate from the ideal angle of 90° (along the AV axis). WT divisions (blue; n=120) favor a division angle of 90° while Mift88 (red; n=161) and MZift88 (green; n=78) mutants show a statistically significant shift to a more randomized division angle, as determined using a Watson-Williams two sample test (WT vs. Mift88 p=<0.001; WT vs. MZift88 p=<0.001; Mift88 vs. MZift88 p=<0.001). (e-f) Lateral views of the mesodermal germ layer in WT embryos expressing Arl13b-GFP (green) and memb-mRFP (red) used to visualize cilia formation during late gastrulation. At 60% epiboly (6hpf) Arl13b-GFP (green) is only observed in the cytoplasm (e), while at 80% epiboly (8.4hpf), it begins to label short cilia that are beginning to form on some cells (yellow arrows; f).
3.4.5 IFT88 is required for PCP-independent OCD during neurulation

To determine whether IFT88 functions upstream or in parallel to PCP, I analyzed whether IFT88 was required for PCP-independent OCD occurring during neurulation. At neural keel stages (12hpf), dividing neural progenitor cells are initially oriented so their spindle poles are aligned with the AP axis of the neural keel and their metaphase plates are perpendicular to the neural midline (Figure 3.4.6a,b). These cells subsequently undergo a rotation so their spindle poles become oriented along the medio-lateral (ML) axis and their metaphase plates are parallel to the midline (Figure 3.4.6c,d) [158]. This rotation ensures that daughter cells divide across the midline and does not require PCP signalling for proper spindle orientation [159].

To analyze whether IFT88 was required for OCD in the neural keel, I labeled WT and MZift88 embryos with Arl13b-GFP (cilia and slight membrane labelling) and histone 2B-Cherry (labels histones) and measured the angle of the metaphase plate in relation to the neural midline (set as 0°) before and after rotation (Figure 3.4.6a-d). I found that the initial orientation of the metaphase plate was not significantly different between WT (n=73) and MZift88 (n=60) mutant neural progenitor cells (Figure 3.4.6e). However, the orientation of the metaphase plate after rotation was significantly disrupted in MZift88 mutants, albeit to a lesser degree than observed for gastrulation OCD (Figure 3.4.6f). These results suggest that IFT88 can control OCD independent of PCP signalling.
Figure 3.4.6 IFT88 is required for PCP-independent OCD during neurulation. (a-d) Confocal images visualizing cell divisions in 6-somite stage (12hpf) WT embryos expressing Arl13b-GFP (green; cell membranes) and histone 2B-Cherry (red; histones). Time-lapse confocal z-series were generated every 3min for 1 hour and representative stills of a dividing cell at different time points display cell orientation at the onset of division (a), the initial orientation of the metaphase plate (b), the orientation of the metaphase plate after rotation (c), and the cell in anaphase (d). Cell orientation before and after rotation is determined by measuring the angle between the metaphase plate and the neural midline (set as 0°). (e) Graph of cell orientation at the onset of metaphase as measured by the percentage of cells that have metaphase plates aligned with the neural midline (0°). The orientation of WT (blue; n=73) and MZift88 (red; n=60) cell divisions are not statistically significant (p=0.830) and their metaphase plates are only slightly aligned with the midline. (f) Graph of cell orientation after the cells have undergone a rotation as measured by the percentage of cells that have metaphase plates aligned with the neural midline (0°). WT cells (blue; n=73) strongly favor an orientation where their metaphase plates are aligned with the midline (0°) while MZift88 cells (red; n=60) exhibit a statistically significant shift (p=0.010) to a more randomized orientation. Statistical significance was determined using a Watson-Williams two sample test and statistical significance was assigned when p <0.05.
3.4.6 IFT88 is not required for astral microtubule formation

I next sought to determine the mechanism by which IFT88 is regulating the orientation of cell division. It has been shown that spindle orientation is largely determined by pulling forces exerted on astral microtubules, which radiate from the spindle poles and anchor them to the cell cortex [160]. A recent study by Delaval et al. (2011) reported that the depletion of IFT88 using siRNA in HeLa cells resulted in mitotic defects. They implicated IFT88 in the formation of astral microtubules by participating in a dynein1-driven complex and transporting peripheral microtubule clusters and microtubule-nucleating proteins [138].

To determine whether IFT88 is required for astral microtubule formation in whole embryos, I performed immunohistochemistry on WT and Mift88/MZift88 (50% Mift88, 50% MZift88) mutant embryos using an antibody against α-tubulin. I observed robust astral microtubule formation in most Mift88/MZift88 cells and astral microtubules were not obviously different from those in WT cells (Figure 3.4.7a,b). I quantified astral microtubule formation according to Delaval et al. (2011) and measured the percentage of cells with long astral microtubules (>3μm) [138]. I found that 100% (n=60) of WT cells had long astral microtubules compared to 98% (n=60) of Mift88/MZift88 cells (Figure 3.4.7c). My observations differ from what was reported in Delaval et al. (2011) where they saw 65% of control cells with long astral microtubules and only 30% when IFT88 was depleted with siRNA [138]. This suggests that function of IFT88 in controlling the formation of astral microtubules in HeLa cells does not hold true for Mift88/MZift88 mutant embryos, and IFT88 may regulate OCD through a different mechanism.
Figure 3.4.7 IFT88 is not required for astral microtubule formation. (a-d)

Immunohistochemical analysis of astral microtubule formation using anti α-tubulin staining (green). (a) A representative WT cell demonstrating robust astral microtubule formation and the presence of long astral microtubules that are greater than 3µm (inset). (b) A representative Mift88/MZift88 cell demonstrating robust astral microtubule formation and the presence of long astral microtubules that are greater than 3µm (inset). (c) Quantification of the percentage of WT (blue, n=60) and Mift88/MZift88 mutant (red, n=60) cells with long astral microtubules (>3µm).
3.5 Discussion

I have successfully utilized a germ-line replacement strategy to eliminate maternal contribution and generate MZift88 mutant embryos. While zygotic ift88 mutants simply have a defect in cilia maintenance, MZift88 mutants have a defect in initial cilia formation, suggesting that IFT is in fact required for cilia formation in zebrafish. My analysis of PCP in MZift88 mutants conclusively demonstrated that PCP-mediated CE movements are normal, despite an absence of the ciliary axoneme. My results are in agreement with Huang et al. (2009) that also report the generation and characterization of MZift88 mutants. They observed disrupted cilia formation in MZift88 mutants, with no defects in CE movements and only a mild defect in Hh signalling [135]. Taken together, analysis of MZift88 mutants suggests that the ciliary axoneme is not required for PCP. Therefore, CE defects observed with the knockdown of other basal body and cilia-associated proteins may not be specific to their ciliary functions [4-8]. However, MZift88 mutants retain apically docked basal bodies with small remnants of the ciliary membrane and I have not excluded the possibility that these structures may be sufficient for PCP. Exactly how much of the cilium is required for various signalling functions remains unclear.

I have also examined the role that cilia-generated fluid flow has on the polarity of primary motile cilia. By examining basal body positioning on the apical surface of floorplate cells in MZift88 mutants, I have determined that cilia-generated fluid flow is not required for the positioning of primary motile cilia. However, I do have some preliminary evidence that ciliary flow is required to reinforce the posterior orientation of cilia. A role for cilia-generated fluid flow in the orientation of primary motile cilia would be consistent with what was observed for multi-ciliated cells of the Xenopus epidermis and the mouse brain [140, 141]. In Chapter 2, I showed that the posterior orientation of motile primary cilia in the ventral neural tube is controlled by PCP signalling. Therefore, defects in cilia orientation in the absence of cilia-generated fluid flow would suggest an indirect role for cilia in re-enforcing the PCP of cells in the ventral neural tube, but further examination is required to fully characterize the defect in MZift88 mutants.

My analysis of MZift88 mutants has provided the first in vivo evidence that IFT88 is required for controlling OCD. Moreover, I have provided direct evidence that this function of IFT88 is independent of its function in cilia formation, since the OCD studied at gastrulation precede initial cilia formation. Subsequent analysis of OCD occurring during neurulation revealed that
IFT88 controls both PCP-dependent (gastrulation) and PCP-independent (neurulation) OCD, suggesting that IFT88 functions parallel to PCP. However, MZift88 mutants do not display any obvious phenotypes that can be attributed to defects in OCD during gastrulation or neurulation, like CE defects or an accumulation of cells at the neural midline [36, 43]. This is likely due to misoriented cells being rescued by the bulk movement of neighboring cells, in response to PCP-mediated CE movements that drive the extension and narrowing of the body axis and the developing neural tube [154]. Interestingly, during kidney tubule development, OCD are believed to contribute to maintaining kidney tubule diameter and MZift88 mutants exhibit kidney cysts, consistent with defects in OCD [98, 100].

I also examined whether disrupted astral microtubule formation was responsible for defects in OCD occurring during gastrulation, which was reported with IFT88 depletion in HeLa cells, [138]. Surprisingly, I found that astral microtubule formation was normal in Mift88/MZift88 mutant embryos since almost all cells formed long astral microtubules. These discrepancies could be due to inherent differences between zebrafish ectodermal cells and HeLa cells. In support of this, it has previously been shown that the tubulin content differs between tissue culture cells and tissues, and the reported lower tubulin content in tissue culture cells may impact the assembly of astral microtubules [161]. Since IFT88 is not regulating astral microtubule formation in zebrafish ectodermal cells, it may be controlling OCD through a different mechanism that remains to be identified.
Chapter 4

4. Discussion and Future Directions

4.1 Summary of Principal Findings

The main objective of my thesis was to elucidate the relationship between cilia and PCP signalling in the context of vertebrate development, and the work I have presented here has contributed significantly to our understanding of how the two are related. My first goal was to develop a marker of cilia that would enable me to answer these questions in a living zebrafish embryo. I generated constructs encoding fluorescently-tagged Arl13b, a protein that localizes along the entire length of the ciliary membrane. Arl13b-GFP is the first reported in vivo marker of cilia and enables the study of cilia structure, orientation, and dynamics. I also developed stable transgenic zebrafish lines that express fluorescently-tagged Arl13b under a ubiquitous β-actin promoter (Tg βact::Arl13b-GFP/Cherry) which have been utilized for diverse purposes by the zebrafish community.

I began by examining the role that PCP signalling plays on the formation of cilia, a notion that primarily stems from studies of multi-ciliated epithelial cells in Xenopus. Two studies by Park et al. (2006, 2008) analyzed the function of the PCP effectors, Inturned and Fuzzy, followed by the Wnt signalling factor, Dvl, and implicated them in regulating an enrichment of apical actin that is essential for basal body docking and cilia formation [2, 3]. Inturned and Fuzzy have been designated as PCP effector proteins, but they are not considered to be specific to PCP signalling or part of the core pathway. Dvl is part of the core PCP pathway, but it is also shared with other branches of Wnt signalling [47]. Therefore, it was unclear whether the function of these factors in cilia formation was a universal feature of all PCP factors and was specific to core PCP signalling. To determine whether core PCP signalling was required for cilia formation, I analyzed the MZvangl2 mutant, a complete loss of function mutant of a core and specific PCP regulator. I found that cilia formation was not disrupted in MZvangl2 mutants, but I observed defects in the uniform posterior positioning and tilting of motile primary cilia. To determine whether this feature was shared with other PCP factors, I examined the function of another PCP regulator, Knypek. Analysis of MZknypek mutants also revealed normal cilia formation, with defects in the positioning and tilting of motile primary cilia. Therefore, based on my analysis of
MZvangl2 and MZknypek mutants, I concluded that core PCP signalling is not required for cilia formation, and defects in the apical docking of basal bodies observed with the knockdown of Inturned, Fuzzy, and Dvl are not specific to their functions in PCP signalling.

To study the reciprocal perspective on the relationship between cilia and PCP signalling, I examined whether cilia were required for PCP. Cilia became implicated in regulating PCP when CE defects were observed with the knockdown of certain proteins that localize at or near cilia or basal bodies including Inversin, BBS 1, 4, 6, Ofd1, Kif3a, and Lrrc6l [4-8, 123]. Surprisingly, mutants of genes that were believed to function exclusively in cilia formation like those encoding IFT proteins were not reported to have CE defects, despite the eventual loss of all cilia [129, 131]. In zebrafish ift88 mutants cilia persist till approximately 4dpf, suggesting that IFT is not required for cilia formation in zebrafish [131]. Since cilia are still present while PCP-mediated CE movements are occurring, it was unclear of what role, if any, cilia had in regulating PCP. The ift88 mutant was shown to retain high levels of maternal ift88 RNA (from heterozygote mothers), which was proposed to rescue cilia formation during early development [162]. In an attempt to generate mutants where cilia formation is completely abolished, I utilized a germ-line replacement strategy to eliminate maternal contribution and generate MZift88 mutants [143]. I found that MZift88 mutants had defects in initial cilia formation where the axoneme is absent in these mutants, indicating that IFT is in fact required for cilia formation in zebrafish. Using different approaches, I analyzed PCP-mediated CE movements in MZift88 mutants and determined that the ciliary axoneme is not required for PCP signal. I also found that fluid flow generated by motile floorplate cilia is not required for the posterior positioning of basal bodies on the apical surface of floorplate cells, but I have evidence that cilia-generated fluid flow may be required to reinforce the orientation of these same cilia. Finally, I provide the first in vivo evidence that IFT88 plays a cilia-independent role in controlling OCD occurring during gastrulation and neurulation, and that this is not due to defects in astral microtubule formation.

4.2 The Role of Vangl2 and Other Core PCP Factors in Cilia Formation May Be Cell-Type Specific

Shortly after our work was published, a study by Song et al. (2010) came out that supported our principal findings. They generated mouse vangl1/vangl2 double mutants and observed normal cilia formation in multi-ciliated airway cells, the neural tube, and cultured MEF cells. They also
observed randomly positioned cilia on the apical surface of posterior notochord cells and reported turbulent nodal flow [115]. However, a subsequent study by May-Simera et al. (2010) examined another vangl2 mutant in zebrafish and showed a reduced number and shorter KV cilia, stemming from a defect in basal body docking [95]. While this conflicts with our findings, there are important distinctions between the two studies. Firstly, there are differences in the methodologies used to visualize cilia since our study utilized Arl13b-GFP labeling in vivo, whereas May-Simera et al. (2010) used an antibody against acetylated tubulin in fixed tissues. Arl13b-GFP labels the ciliary membrane while acetylated tubulin staining labels only stable microtubules, and it is possible that vangl2 mutants have less stable cilia [163]. Secondly, the two studies analyzed different mutant alleles of vangl2. Our study utilized the tri95 mutant allele that results in the deletion of the open reading frame, and is thought to be a null because no vangl2 transcript was detected in homozygous mutant embryos [27]. Their study utilized the tri209 mutant allele that results in a premature termination of translation in the last exon [27]. A recent study comparing the phenotypes of different vangl2 mouse mutants showed that the looptail (Vangl2S464N) mutation, a serine to asparagine substitution that occurs near the C-terminal end of the protein, affects binding to Dvl and exhibits dominant and neomorphic activity [117, 164]. It is possible that the tri209 allele may also encode a protein that behaves as a neomorph, in which case the cilia formation defects observed with this allele may not represent a vangl2 loss-of-function phenotype.

Another study by Tissir et al. (2010) examined the role of the core PCP factor Celsr on mouse brain development. They generated celsr2/celsr3 double mutants and reported defects in basal body docking and cilia formation in multi-ciliated ependymal cells, but primary cilia present in the choroid plexus were found to be unaffected [111]. It is possible that the function of core PCP factors in cilia formation is cell-type specific. PCP signalling may be required for basal body docking and cilia formation in some specialized cells but not in others. This is supported by the analysis of Vangl2 function in Xenopus where the same group first reported that vangl2 morphants had a reduced number of apically docked basal bodies in multi-ciliated epidermal cells, and then later reported that nodal primary cilia were unaffected in these morphants [58, 113]. This discrepancy is not surprising since there are significant differences in the biogenesis of primary cilia and those present on multi-ciliated cells. In cells with primary cilia, new basal bodies form adjacent to preexisting ones, whereas in multi-ciliated cells there is a de novo
mechanism that can generate hundreds of basal bodies simultaneously [165]. However, multiciliated epithelial cells were still observed in the pronephric duct of MZvangl2 mutants.

An alternative explanation for why cilia formation defects have been reported with vangl2 knockdown in some tissues but not others is that Vangl2 may actually be required for cilia maintenance, and different tissues may exhibit varying susceptibilities to ciliary degeneration. Vladar et al. (2012) examined vangl1 conditional knockout mice and observed some multiciliated epidermal cells with shorter and sparser cilia. Surprisingly, basal bodies were still apically docked and Dvl was properly localized, suggesting that early mechanisms involved in cilia formation are normal [118]. These observations are consistent with a defect arising from cilia degeneration, however the long-term affects of PCP signalling on cilia maintenance have not been explored. Preliminary evidence suggesting that MZvangl2 mutants have defects in striated rootlet formation would provide a possible mechanism for how Vangl2 could be regulating cilia maintenance (refer to section 4.5).

4.3 Possible Explanations for the CE Defects Observed with the Knockdown of Ciliary Proteins

In Chapter 3, I conclusively determined that the ciliary axoneme is not required for CE movements and my results are in agreement with Huang et al. (2009), which also reported normal extension of the body axis in MZift88 mutants [135]. I also examined MZcep290 mutants to determine whether the absence of CE defects in MZift88 mutants is shared with other cilia-specific mutants. MZcep290 mutants have cilia-associated defects including a curved body axis and kidney cysts, and this is due to the function of Cep290 at the transition zone, where it is believed to serve a gatekeeper role and regulate the trafficking of ciliary proteins [166]. I examined whether CE movements were disrupted in MZcep290 mutants by analyzing the extension of the body axis using krox20 (WT n=8, MZcep290 n=10) and myoD (WT n=7, MZcep290 n=10), and the migration of the prechordal plate using ntl, hgg1, and dlx (WT n=7, MZcep290 n=5) RNA in situ hybridization. Similar to what was observed with MZift88 mutants, there were no significant differences in either body axis extension or prechordal plate migration between WT and MZcep290 mutants (Figure 4.3.1a-g).

In light of these observations, it suggests that the CE defects resulting from the knockdown of certain ciliary proteins are not specific to their ciliary functions. Many of these proteins function
in several cellular compartments where they serve other functions like regulating cell adhesion and polarized microtubule dynamics, and it is possible that these non-ciliary functions are actually responsible for regulating CE movements [125-127]. Also, CE defects were observed using MO-mediated knockdown and MOs are notorious for causing off-target and toxic effects [167].

Another possibility is that the basal body is the key area of the cilium that is required for PCP. TEM revealed that while the axoneme is absent in MZif88 mutants, basal body docking at the apical membrane is perfectly intact, and small patches of the ciliary membrane remain. While the mechanosensory function attributed to cilia would be disrupted in MZif88 mutants, the basal body may be sufficient for PCP. In fact, Inversin, BBS 1, 4, 6, and Ofd1 all localize to the basal body, suggesting that the switch between canonical Wnt and PCP signalling may be controlled at the basal body instead of the axoneme, but further examination will be necessary to distinguish between the various ciliary compartments, and to determine the functions of the basal body [4, 5, 7, 8].

Alternatively, cilia may still play a role in regulating the switch between canonical Wnt and PCP signalling but this role would likely be subtle, and be buffered in the embryo as a whole, since MZif88 mutants do not display any phenotypes consistent with hyperactive canonical Wnt (double axis) or disrupted PCP signalling (CE defects) [135]. Over-active canonical Wnt signalling has been reported with the knockdown of ciliary proteins in cell culture or in specific tissues like the mammary epithelium [5, 123, 133]. Thus, if cilia are required to regulate Wnt signalling, it is likely to be cell-type specific or be limited to specific times during development.
Figure 4.3.1 MZcep290 mutants have normal CE movements. (a-d) Whole-mount flat-mount RNA *in situ* hybridization showing representative 10-somite stage WT (a) and MZcep290 (b) embryos with krox20 and myoD riboprobes. Quantification of the medio-lateral extent of krox20 (c; p=0.96) and the anterior posterior extent of myoD expression (d; p=0.34) demonstrate no significant differences in CE of the body axis between WT (n=8, krox20; n=7, myoD) and MZcep290 mutants (n=10). (e-g) Whole-mount RNA *in situ* hybridization showing representative bud-stage WT (e; n=7) and MZcep290 (f; n=5) embryos with ntl, hgg1, and dlx riboprobes. Ntl, hgg1, dlx expression demonstrate normal migration of the prechordal plate in MZcep290 and quantification of the gap between the prechordal plate and the notochord (g) revealed no significant differences (p=0.26). Statistical significance for all experiments was determined using a 2-tailed student T-test and alpha was set as 0.05.
4.4 Determining the Mechanism by which Vangl2 Controls the Positioning of Primary Motile Cilia

Ultimately my goal is to determine the mechanism by which Vangl2 is controlling the positioning and orientation of primary motile cilia. PCP signalling is believed to induce polarity by acting on the actin cytoskeleton. In the *Drosophila* wing, each epithelial cell has a hair growing from the distal end of the apical surface. Hair growth is initiated by the asymmetric localization of PCP components and the preferential enrichment of actin on the distal side of the cells [16]. Since this system resembles the uniform posterior positioning of cilia, it suggests that a similar mechanism involving actin enrichment may also specify the location of basal body docking on the surface of floorplate cells. In support of this, defects in basal body docking observed with the knockdown of Inturned, Fuzzy, and Dvl in *Xenopus* epithelial cells were attributed to a thinning of the apical actin cap [2, 3]. *Xenopus* epithelial cells are multi-ciliated so basal bodies dock along the entire apical surface, but in floorplate cells the situation is different because they are mono-ciliated, with a single motile cilium emanating from the posterior apical surface. If actin enrichment is involved in the polarized docking of basal bodies in floorplate cells, it should be present on the posterior side of these cells. Traditionally, actin is visualized using phalloidin staining, an agent that binds to and stabilizes F-actin. However, phalloidin is not effective for imaging actin dynamics in living cells and would not capture actin accumulations that were only transient. Recent work has developed mCherry-UtrCH as an *in vivo* marker of F-actin that does not interfere with its dynamics [168]. I will use this marker to study actin dynamics as floorplate cells develop and form cilia to determine if the site of ciliogenesis corresponds to a site of actin enrichment, and to determine if this enrichment is lost in MZ*vangl2* mutants (Figure 4.4.1a). I can also utilize actin drug inhibitors like Cytochalasin-D and Latrunculin-B on live WT embryos at stages when the floorplate is developing to see if basal body polarity is disrupted upon treatment [169]. I also plan to study the distribution of factors downstream of core PCP signalling, like the Rho GTPases, which have been shown to modify the actin cytoskeleton [170, 171]. I can use fluorescent markers like RhoaCT32-GFP (labels RhoA) to determine whether these factors accumulate at the posterior apical side of floorplate cells, corresponding to the location of basal body docking, and whether this localization is disrupted in MZ*vangl2* mutants (Figure 4.4.1a) [172].
I will also examine whether the microtubule cytoskeleton is involved in the polarized positioning of basal bodies. Previous studies have shown that microtubules regulate the early establishment of PCP, where PCP components are transported asymmetrically along microtubule arrays [128].

There is also some evidence that microtubules play a role downstream of PCP factor localization. Vladar et al. (2012) examined multi-ciliated epithelial cells of the mouse airway and identified a planar polarized microtubule network that first appears at the time of basal body docking. They visualized an enrichment of tyrosinated microtubules at the proximal side of the cell cortex, in a region close to where Fz/Dvl crescents are localized. This polarized stabilization of microtubules requires PCP signalling and was shown to be required for the planar polarity of airway cilia [118]. I will utilize anti-tyrosinated α-tubulin antibody labelling to test whether these polarized microtubule arrays are also present in mono-ciliated floorplate cells, and if so, I will treat embryos with low doses of Nocodazole (microtubule depolymerizing agent) to see if basal body positioning requires polarized microtubules (Figure 4.4.1b).
Figure 4.4.1 Schematics representing possible mechanisms for the posterior positioning of basal bodies on the apical surface of floorplate cells. (a) Localization of downstream PCP effectors like Rho-GTPases to the posterior end of the apical surface may modify the actin cytoskeleton and result in an enrichment of actin that is required for basal body docking and cilia formation (left). MZvangl2 mutants may lack this posterior actin enrichment resulting in basal bodies that are randomly docked at the apical membrane (right). (b) Polarized microtubule arrays may be required for the asymmetric distribution of PCP components and tyrosinated microtubules may specify the location of basal body docking (left). MZvangl2 mutants may have defects in the organization of intracellular microtubule arrays, preventing the initial establishment of cell polarity and polarized basal body docking (right).
4.5 Determining the Mechanism by which Vangl2 Controls the Tilting of Primary Motile Cilia

As noted in Chapter 2, MZvvangl2 mutants exhibit defects in the posterior tilting of primary motile cilia in KV, the ventral neural tube, and the pronephric duct. Interestingly, when MZvvangl2 mutant cells were transplanted into the ventral neural tube of WT hosts, I observed instances where mutant cilia swayed, suggesting that there may be a defect in their anchoring, since cilia swaying was not observed in WT to WT transplants. To gain a better understanding of the docking and anchoring of basal bodies at the apical membrane of floorplate cells, I performed TEM on both WT and MZvvangl2 mutant embryos at 30hpf. In WT embryos, I observed apically docked basal bodies with basal feet and striated rootlets visibly emanating from basal bodies (asterisk; Figure 4.5.1a,c). In MZvvangl2 mutants I also observed apically docked basal bodies and associated basal feet, but I did not observe the presence of striated rootlets (Figure 4.5.1b,d). Single micrograph sections of individual basal bodies revealed striated rootlets on 71% of WT basal bodies (n=14) but were never observed on MZvvangl2 basal bodies (n=7), (Figure 4.5.1e). While the exact function of the striated rootlets is unclear, they are believed to interact with the cytoskeleton and provide structural support for the cilium, which was intriguing given the defects in the anchoring of motile cilia in MZvvangl2 mutants [173].

The major structural component of striated rootlets is the protein Rootletin that bundles into thick, striated filaments [174]. Yang et al. (2005) generated rootletin mutant mice to determine the role of striated rootlets in cilia formation and function. Despite the complete absence of striated rootlets in rootletin mutants, they still form motile cilia, but have disrupted ciliary flow and insufficient mucociliary clearance. Mutants also exhibit a degeneration of photoreceptor cilia, likely due to increased sensitivity to mechanical stress [173]. A recent study by Werner et al. (2011) shed some light onto how striated rootlets interact with the underlying cytoskeleton. They examined Xenopus multi-ciliated epithelial cells and reported two distinct pools of cortical actin. Apical actin is located in the same plane as basal bodies and it forms a mesh-like network, whereas sub-apical actin is located below apical actin and it links the basal body of one cilium to the striated rootlet of its neighbor. They identified a dose of Cytochalasin D (inhibits actin polymerization) that specifically targets the sub-apical pool of actin and found that this disrupted global coordination of cilia polarity. Interestingly, when they performed TEM, they found that
microtubules are also enriched at striated rootlets and are required for local coordination of cilia polarity [175].

In the context of mono-ciliated floorplate cells, the interactions of striated rootlets with the actin and microtubule cytoskeletons is probably quite different, but here too they are likely to play an important role in regulating cilia polarity. When basal bodies dock at the posterior end of the apical membrane, their striated rootlets may attach to an assembly of actin that is similar to the sub-apical actin pool identified in multi-ciliated epithelial cells. The pulling forces generated by this interaction on the anterior side of basal bodies may be required to tilt basal bodies and thus cilia, in the posterior direction. The interaction of striated rootlets with the actin cytoskeleton may also anchor and provide structural support for motile cilia, so they are better able to resist the mechanical stress of ciliary beating (Figure 4.5.2a). If striated rootlets are disrupted or absent, it would likely result in misoriented cilia that would have a tendency to sway, both of which are observed in MZvangl2 mutants (Figure 4.5.2b).

Since TEM analysis involves single micrograph sections, it is possible that striated rootlets are still present, but are not visible in the specific plane of individual basal bodies. Seeing as I cannot conclude that rootlets are absent based on TEM alone, I will try visualizing them by other methods. CLAMP-GFP (calponin-homology and microtubule associated protein) has previously been shown to label striated rootlets and I can use this marker to test whether striated rootlets are still present in MZvangl2 mutants [3]. Preliminary observation of CLAMP-GFP expression in WT embryos shows an enrichment of CLAMP-GFP at the base of cilia in floorplate cells, consistent with it labelling striated rootlets (Figure 4.5.3). I can also inject a MO against zebrafish rootletin to inhibit striated rootlet formation and see if it phenocopies the defect in cilia tilting and swaying observed in MZvangl2 mutants [24].

Disrupted striated rootlet formation may also be responsible for the defects in cilia number that have been observed with the knockdown of some core PCP proteins in certain tissues [58, 111, 113, 118]. Defects in cilia number may be due to cilia degeneration since striated rootlets have been shown to be required for the maintenance of photoreceptor cilia [173]. Certain tissues like multi-ciliated cells may be more susceptible to ciliary degeneration, whereas primary motile cilia may not be as susceptible. Conditional mutants will be useful to determine the long-term roles of
core PCP factors on cilia maintenance, which have not been explored due to the embryonic lethality of PCP mutants.
Figure 4.5.1 Striated rootlets are absent in MZvangl2 mutants. (a,b) TEM performed on 30hpf WT (a) and MZvangl2 mutant (b) embryos. Pictures show transverse sections of basal bodies docked at the apical membrane of cells in the ventral neural tube. (c,d) Coronal views of WT (c) and MZvangl2 mutant (d) basal bodies on floorplate cells. WT basal bodies display the presence of both basal feet and striated rootlets (asterisk; a,c), but in MZvangl2 mutant basal bodies, striated rootlets were not observed (b,d). (e) Table indicating the percentage of WT (n=14) and MZvangl2 mutant (n=7) basal bodies imaged that were associated with both striated rootlets and basal feet, basal feet alone, and rootlets alone.
Figure 4.5.2 Schematic representing a possible mechanism for the posterior orientation of basal bodies on the apical surface of floorplate cells. (a) When basal bodies dock at the posterior apical membrane of WT cells, their striated rootlets associate with a sub-apical pool of actin that generates pulling forces on the anterior side of the basal body, causing it to tilt in the posterior direction. The association of striated rootlets with the sub-apical actin subsequently serves to anchor the motile cilium, helping it to resist the mechanical stress of ciliary beating. (b) In MZvangl2 mutant floorplate cells, the striated rootlets may be disrupted or absent. This results in cilia that are misoriented and sway because they are not anchored in by the interaction of striated rootlets with sub-apical actin.
Figure 4.5.3 CLAMP-GFP labels the striated rootlets of floorplate cilia.

Coronal views of WT embryos expressing CLAMP-GFP (green; rootlets) and memb-RFP (red; cell membranes) with the developing floorplate visible at 14-16 somites. Enrichment of CLAMP-GFP at the posterior side of floorplate cells is consistent with it marking striated rootlets (yellow arrows; inset).
4.6 Further Characterizing the Role for Cilia-Generated Fluid Flow in Regulating the Orientation of Basal Bodies

In Chapter 3 I describe some preliminary evidence that cilia-generated fluid flow is required to reinforce the orientation of motile cilia on the surface of floorplate cells. To fully characterize this defect, I will need to repeat experiments where I transplant Arl13b-GFP labelled WT cells into unlabelled MZift88 hosts to see if my initial observation of floorplate cilia swaying holds true. My finding that cilia-generated fluid flow may be required for the orientation of primary motile cilia is consistent with observations in multi-ciliated cells, suggesting that similar mechanisms are at play [140, 141]. However, cilia-generated fluid flow was shown to establish cilia orientation in multi-ciliated cells, whereas I have shown that it may be required to reinforce the orientation of primary cilia on floorplate cells. Since MZift88 mutants have defects in axoneme formation, I cannot study cilia orientation directly. To overcome this, I will use CLAMP-GFP to label striated rootlets in MZift88 mutants, since rootlets can be used to visualize the orientation of basal bodies even when the ciliary axoneme is not present [3]. Striated rootlets project from the basal body in an orientation opposite to the direction that cilia are oriented in [3]. So in the case of floorplate cells, if basal bodies are properly posteriorly oriented in MZift88 mutants then I would expect striated rootlets to be projecting in the anterior direction (left; Figure 4.6.1a). However, if cilia-generated fluid flow is required for the orientation of basal bodies, then striated rootlets will project with a random orientation (right; Figure 4.6.1a).

As noted above, some cilia on MZvangl2 mutant cells were also observed to sway, and I hypothesized that this may be due to defects in striated rootlet formation. The fluid flow generated by motile cilia themselves may exert forces that are transmitted through the ciliary axoneme to striated rootlets. Striated rootlets may subsequently interact with the cytoskeleton in order to tilt and anchor motile cilia in the proper orientation (Figure 4.6.1b). This model is one possible explanation of why cilia would sway when both cilia-generated fluid flow and striated rootlets are disrupted, but clearly this topic warrants further examination.
Figure 4.6.1 Strategy for determining whether cilia-generated fluid flow is required for cilia orientation. (a) MZift88 mutant embryos will be labeled with Cherry-Centrin (red; basal bodies) and CLAMP-GFP (green; striated rootlets) to assess the orientation of basal bodies docked at the apical surface of floorplate cells. If cilia generated fluid flow is not required to orient basal bodies then striated rootlets will uniformly project in the anterior direction in MZift88 mutants (left). If cilia-generated fluid flow is required to orient basal bodies then floorplate cells in MZift88 mutants will exhibit a randomization in the direction that striated rootlets project in from basal bodies (right). (b) Cilia-generated fluid flow may reinforce the orientation of primary motile cilia on floorplate cells. Fluid flow may exert mechanical forces that get transmitted through the ciliary axoneme and induce an intracellular response that promotes the interaction of striated rootlets with sub-apical actin, required to anchor and reinforce the tilting of floorplate cilia.
4.7 Determining whether IFT88 Controls OCD through the Gai-LGN-NuMA Complex

Since I have shown that the formation of astral microtubules is not disrupted in MZift88 mutants, IFT88 may regulate spindle orientation through another mechanism. There are many different factors that regulate OCD including extrinsic signals like those from cell-extracellular matrix adhesion or cell-cell contacts, as well as intrinsic signals like those from polarity proteins [176]. Of these factors, the Gai-LGN-NuMA complex is the most obvious candidate because it has been shown to regulate OCD occurring during zebrafish gastrulation and chick neurulation [177, 178]. The Gai-LGN-NuMA complex localizes asymmetrically at the cell cortex during metaphase and is required for spindle orientation because it links astral microtubules to the cell cortex [179]. The Gai-LGN-NuMA complex is linked to the cell membrane through the myristylation of Gai and NuMA mediates its interaction with both astral microtubules and the dynein-dynactin microtubule motor complex [180, 181]. The tethering of astral microtubules to the cell cortex combined with the minus-end directed motor activity of the dynein-dynactin complex, generates unequal pulling forces on astral microtubules that direct spindle positioning and rotation (inset; Figure 4.7.1) [179]. The precise localization of Gai-LGN-NuMA is essential for proper spindle positioning and its knockdown or mislocalization leads to misoriented cell division [182]. In HeLa cells, Gai-LGN-NuMA is restricted to the lateral cortex during metaphase by a chromosome-derived gradient of RanGTP that excludes it from the midzone. Additionally, spindle-pole derived Plk1 phosphorylation causes the dynein-dynactin complex to dissociate from Gai-LGN-NuMA, thus adjusting pulling forces when the spindle pole is in close proximity to the cell cortex (left; Figure 4.7.1) [183]. However in chick neuroepithelial cells, Gai, LGN, and NuMA were not found to be asymmetrically localized, but were instead reported to localize in a homogenous belt along the lateral cell cortex [178].

Segalen et al. (2010) examined the function of NuMA in orienting epiblast cell divisions during zebrafish gastrulation. They report that in epiblast cells NuMA also localizes along the cell cortex in approximately half of cells, but remains cytoplasmic in others. They used a MO to induce NuMA partial loss of function and observed misoriented cell divisions, but reported that spindle morphology was unaffected. Interestingly, they found that the Gai-LGN-NuMA complex was downstream of the PCP pathway, where Dvl binds the C-terminal domain of NuMA and NuMA is required for Dvl-mediated spindle orientation. A link between the PCP pathway and
the G\(\alpha\)-LGN-NuMA complex provides mechanistic insight into how extrinsic polarity cues control cell intrinsic cues that mediate mitotic spindle orientation during gastrulation [177].

IFT88 functioning upstream of G\(\alpha\)-LGN-NuMA localization seemed like a possible mechanism to explain OCD defects in \(\text{MZ}\mbox{-ift88}\) mutants since the G\(\alpha\)-LGN-NuMA complex functions in tissues where IFT88 has also been shown to regulate OCD. Also, defects in G\(\alpha\)-LGN-NuMA localization phenocopy IFT88 loss of function, where cell divisions are misoriented, but spindle poles and astral microtubules are not disrupted. To determine whether IFT88 is required for G\(\alpha\)-LGN-NuMA complex localization, I will study the distribution of these factors in relation to spindle orientation in \(\text{MZ}\mbox{-ift88}\) mutants using the expression of fluorescently-tagged proteins and antibodies when available. I examined GFP-LGN localization in WT neuroepithelial cells (6-somite stage) and found that at low levels (40pg of GFP-LGN RNA) GFP-LGN is asymmetrically enriched at the cell cortex in dividing cells (arrow; Figure 4.7.2a). The enrichment corresponds to the positioning of spindle poles and is consistent with the asymmetric pattern observed in HeLa cells [183]. At higher levels, or in epiblast cells during gastrulation, GFP-LGN localizes along the entire cell cortex in dividing cells (arrow, Figure 4.4.2b). Differences in the localization pattern of GFP-LGN are likely due to RNA turnover at later stages, whereas at earlier stages GFP-LGN may be over-saturated, since uniform cortical localization of LGN would lead to random spindle orientation based on the model proposed in HeLa cells [183]. Further studies will be required to titrate the levels of GFP-LGN in different cell types before I can study its distribution in \(\text{MZ}\mbox{-ift88}\) mutants.

One way that IFT88 may regulate OCD is that it may be involved in G\(\alpha\)-LGN-NuMA complex trafficking. A complex of IFT proteins that includes IFT88 has been previously shown to regulate intracellular trafficking, where it drives the polarized recycling of endosome-localized TCR/CD3 complexes to specific regions of the cell membrane in non-ciliated cells [139]. Similarly, IFT88 may be involved in establishing the asymmetric distribution of the G\(\alpha\)-LGN-NuMA complex by regulating its polarized targeting at the cell membrane. Additionally, IFT88 may regulate G\(\alpha\)-LGN-NuMA complex distribution through its function at the centrosome where it has been shown to affect G1-S transition in proliferating cells [137]. IFT88 may somehow affect spindle-derived signals like Plk1 phosphorylation, affecting dynein-dynactin association with G\(\alpha\)-LGN-NuMA that is required for proper OCD.
Figure 4.7.1 Gαi-LGN-NuMA complex localizes asymmetrically at the cell cortex and is required for spindle orientation. The Gαi-LGN-NuMA complex localizes to the cell cortex during metaphase and is required to link astral microtubules to the cell cortex. The Gαi-LGN-NuMA complex is linked to the membrane through the myrostalation of Gαi and NuMA connects it to both astral microtubules and the dynein-dynactin motor complex. The minus-end directed motor activity of dynein-dynactin and the tethering of astral microtubules generates pulling forces that regulate spindle positioning (inset; right). Localization of Gαi-LGN-NuMA is restricted to the lateral cell cortex by a chromosome-derived gradient of RanGTP that excludes it from the midzone, and by spindle pole-derived Plk1 which causes dynein-dynactin to dissociate from Gαi-LGN-NuMA so that pulling forces are adjusted when the spindle approaches the cell cortex (left).
Figure 4.7.2 GFP-LGN is asymmetrically expressed at the cortex of dividing neuroepithelial cells. (a,b) Confocal images of WT embryos expressing 40pg of GFP-LGN (green; LGN) and histone 2B-Cherry (red; histones) at neurulation (a) and gastrulation (b). Neuroepithelial cells exhibit cortical enrichment at regions that correspond to spindle pole position in dividing cells (arrow; a), whereas epiblast cells exhibit a homogenous cortical enrichment in dividing cells that is likely due to over-saturation (arrow; b).
4.8 Concluding Remarks

The importance of studying the relationship between cilia and PCP signalling is perhaps best highlighted in the context of PKD. PKD is characterized by dilations in collecting tubules which lead to cystic and enlarged kidneys. Normal tubule diameter is thought to be regulated by CE movements and OCD, both processes controlled by PCP signalling [184]. CE movements drive cell intercalations that lead to the lengthening and narrowing of kidney tubules, while OCD ensure that subsequent proliferation occurs in the proper direction [98, 100, 185]. Even though phenotypic analysis suggested that disrupted PCP signalling was the underlying cause of PKD, genetic studies of human PKD revealed mutations in genes that encode cilia and basal body proteins, and a model was established were cilia play a mechanosensory role in maintaining homeostasis in kidneys [186]. The fact that both PCP signalling and cilia play a role in PKD was explained by either PCP signalling being required for cilia formation, or by cilia being required for PCP signalling [11].

The work I have presented in my thesis conclusively determined that core PCP signalling is not required for cilia formation, and conversely, cilia are not required for PCP. In light of these findings, it now appears that rather than one being required for the other, instead they are functioning in parallel pathways with respect to PKD. PCP signalling may first regulate CE movements required for kidney tubule elongation, and then later it may act to maintain kidney architecture by controlling OCD. Cilia may function as mechanosensors that are essential for kidney tubule development, and then certain ciliary factors like IFT88 may function in controlling OCD in a cilia-independent manner.
References


