Regulation of planar cell polarity
and Vangl2 trafficking by Tmem14a

by

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A thesis submitted in conformity with the requirements
for the degree of Masters of Science
Department of Molecular Genetics
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Abstract

Planar cell polarity (PCP) refers to the coordinated orientation, movement, or structure of cells within the plane of a tissue. Zebrafish PCP mutants such as the vangl2 mutant exhibit defects in convergent extension, neural tube morphogenesis, and ciliary positioning. Tmem14a is a putative tetraspanin protein that was identified as a potential interactor of Vangl2 in a membrane yeast-two hybrid screen. GFP-tagged versions of Tmem14a are localized to the trans-Golgi network in zebrafish neuroepithelial cells. Knockdown of Tmem14a activity results in convergent extension defects, an ectopic accumulation of cells in the neural tube, and disorganized cilia. The localization of GFP-tagged Tmem14a to the trans-Golgi network suggested that Tmem14a plays a role in the trafficking of core PCP components to the cell membrane. Indeed, the membrane localization of GFP-Vangl2 was disrupted in Tmem14a morphants. In HEK293T cells, Tmem14a-GFP expression is polarized towards one side of the cell. Furthermore, overexpression of GFP-Vangl2 causes Golgi scattering, which is rescued by co-expression of Tmem14a-myc. This suggests that GFP-Vangl2 and Tmem14a-myc have opposing functions in regulating Golgi structure and morphology. Thus, Tmem14a is an interactor of Vangl2 and a novel regulator of vertebrate planar cell polarity signaling.
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Chapter 1
Introduction

1 Introduction

1.1 Overview

The coordinated movement and orientation of cells at an axis orthogonal to the plane of a tissue is called planar cell polarity (PCP)\(^1,2\). The PCP pathway is regulated by a set of proteins termed the “core PCP components” that are evolutionarily conserved between Drosophila and vertebrates\(^1,2\). PCP regulates the orientation of hairs and bristles on the Drosophila wing, abdomen and thorax\(^3,4\), and convergent extension movements during vertebrate gastrulation and neurulation\(^5\). In addition, PCP signaling regulates the orientation of cilia in various tissues in vertebrates, and it has been demonstrated that the PCP-specific core component Vangl2 plays an essential role in directing the polarization of cilia on the cell surface\(^6,7\). To further understand the role of Vangl2 in PCP signaling and cilia regulation, a membrane yeast two-hybrid screen was performed and an uncharacterized protein, Tmem14a, was identified as a potential interactor of Vangl2\(^8\). Tmem14a is a putative member of the tetraspanin protein family, a group of proteins that are ubiquitously expressed and are involved in various vesicular trafficking processes in the cell\(^9\). The objective of my thesis is to characterize Tmem14a as a putative interactor of Vangl2 and a novel vertebrate-specific regulator of PCP signaling. Here I compare the differences between vertebrate and invertebrate PCP and review evidence for the regulation of cilia by PCP signaling. I review what is known about Vangl2 function through its interaction with different proteins and describe the role of the trans-Golgi network (TGN) in polarized protein trafficking. Finally, I focus on the role of the tetraspanin protein family in regulation of cilia.

1.2 Planar cell polarity

Planar cell polarity refers to the coordinated orientation, movement, or structure of cells within the plane of a tissue\(^1,2\). PCP is a property that is exhibited in both invertebrate and
Figure 1. Planar cell polarity regulates the uniform orientation of cellular structures such as cilia, fur, and bristles.
vertebrate species\textsuperscript{1,2}. For example, PCP regulates the orientation of hair cells across the proximal/distal axis of the Drosophila wing such that the growth of hairs point uniformly in one direction\textsuperscript{3,4} (figure 1). In mice and zebrafish, directed cell migration and cell intercalation during convergent extension is under the control of PCP\textsuperscript{5,10}. The intercalation of cells that undergo narrowing in the medial/lateral axis results in the lengthening of the embryo in the anterior/posterior direction. Most importantly, PCP signaling generates asymmetry during embryogenesis. In both mice and zebrafish, the position and orientation of cilia in the node or Kupffer’s vesicle creates fluid flow that is required for the breaking of symmetry in the embryo and to generate left/right asymmetry\textsuperscript{6,7}.

The PCP signaling pathway is regulated by a set of core PCP components that are evolutionarily conserved between species\textsuperscript{1,2}. These PCP components include the transmembrane proteins Strabismus/Van Gogh (Stbm/Vang), Frizzled, and Flamingo, and the cytoplasmic components Diego, Prickle and Dishevelled. Molecular interactions between the core PCP components regulate the activity of Dishevelled, which activates downstream signaling pathways that regulate actin cytoskeleton rearrangement in the cell\textsuperscript{11}. This is through the activation of downstream effectors such as RhoA and Rho kinase\textsuperscript{11} (figure 2). Structural changes in the actin cytoskeleton and microtubule network induces cell migration and tissue morphogenetic processes\textsuperscript{11}.

1.3 Planar cell polarity in Drosophila

In Drosophila, PCP regulates the orientation of sensory bristles across the proximal/distal axis of the wing and the posterior/anterior axis of the abdomen and thorax, as well as the orientation of ommatidia and arrangement of photoreceptors within the compound eye\textsuperscript{1-4}. Mutations in core PCP components result in similar phenotypes such as the misalignment of ommatidia and the misorientation of hairs and bristles\textsuperscript{1-4}. The Stbm gene was identified in Drosophila using X-irradiation as a mutagen to uncover mutants in eye development\textsuperscript{4}. Stbm mutants are characterized by their rough eye phenotype\textsuperscript{4}. In wild-type flies, ommatidia are normally arranged in parallel rows; a mutation in Stbm results in the misalignment in the rows of ommatidia\textsuperscript{4}. Stbm mutants also display misoriented bristles on the leg and thorax\textsuperscript{4}. The Flamingo gene was isolated in a γ-ray mutagenesis screen to identify genes involved in tissue polarity\textsuperscript{3}. 
Flamingo mutants exhibit phenotypes similar to Frizzled and Disheveled mutants. For example, in wild type flies, wings have hairs that grow from the distal end of the cell and point distally whereas Flamingo mutants have hair that grow on centrally and some cells have two hairs. The asymmetric localization of the core PCP components in the cell have been well characterized in Drosophila. Stbm/Vang and Prickle are localized to one side of the cell membrane whereas Frizzled, Dishevelled, and Diego are localized to the opposite side (figure 3). In cells of the Drosophila wing, the core PCP components are first recruited to the apical membrane, and then are localized to the proximal or distal membrane. Recruitment to the apical membrane is essential for the polarization of the PCP components in the proximal/distal axis. The transmembrane components Vang, Frizzled, and Flamingo are essential for the apical recruitment of the PCP components, though the cytoplasmic components Prickle, Dishevelled, and Diego are not required. Vang and Prickle are then localized to the proximal membrane whereas Frizzled, Dishevelled, and Diego are localized to the distal membrane. Flamingo is recruited to both the proximal and distal membrane and stabilizes these complexes.

Molecular interactions between the core PCP components have also been well characterized in Drosophila (figure 3). Vang recruits Prickle to the proximal membrane which results in downstream signaling that inhibits the activity of Dishevelled. In turn, the recruitment of Diego to Frizzled at the distal membrane inhibits Prickle activity, which allows for the recruitment of Dishevelled by Frizzled. The PDZ domain of Dishevelled is important for its interaction with the C-terminal tail of Frizzled. Prickle and Diego both compete for binding to Dishevelled; Prickle can inhibit Dishevelled activity by inhibiting the recruitment of Dishevelled to the membrane. Diego has an opposing role to Prickle by also binding to Dishevelled and increasing its localization at the membrane. In addition, Diego can increase the stability of Dishevelled binding to Frizzled.

The asymmetric localization of the core PCP components allows PCP signaling to be propagated across tissues, and cell-cell communication through molecular interactions between the core PCP components can induce planar cell polarity signaling across neighbouring cells. For example, Flamingo-Frizzled and Frizzled-Vang may interact across cell membranes to transduce PCP signaling between cells. Cell clones that express mutated versions of core PCP components can induce aberrant planar cell polarity signaling in neighbouring wild-type cells, resulting in changes in cell orientation. This is termed “domineering nonautonomy.”
Figure 2. The vertebrate PCP signaling pathway. Frizzled and Vangl2 are core PCP components that regulate the recruitment of Dishevelled to the membrane. Glypican/Knypek is a co-receptor of Frizzled and Wnt5/Wnt11 are Frizzled ligands. Ligand binding results in activation of the PCP pathway. Downstream signaling through Dishevelled activates RhoA and ROK, which causes changes in cell polarity, cell adhesion, and cytoskeletal rearrangement. Vangl2 associates with Prickle at the membrane and prevents the recruitment of Dishevelled to the membrane, blocking activation of the PCP pathway.
Figure 3. The core PCP components are asymmetrically localized in Drosophila and molecular interactions have been characterized. Stbm/Vang associates with Prickle at the membrane; Dishevelled and Diego both associate with Frizzled. Diego can inhibit the association of Stbm/Vang with Prickle whereas Prickle can inhibit the association of Frizzled with Dishevelled. Flamingo stabilizes these interactions. (from Seifert and Mlodzik, 2007).
addition, cell clones expressing a version of Frizzled with a loss of function can instruct
neighbouring cells to reorient hair growth towards the clone; expressing a version of Frizzled
with a gain of function can instruct neighbouring cells to reorient hair growth away from the
clone\textsuperscript{1,20}. The function of Frizzled and Stbm/Vang are mutually antagonistic. This is
demonstrated by experiments in which overexpression of Frizzled in cell clones in the wing
causes wing hairs to reorient the direction in which they are pointing away from the clone
whereas overexpression of Stbm/Vang causes wing hairs toward the overexpressing clone\textsuperscript{1,20}.

1.4 Vertebrate planar cell polarity

In vertebrates, PCP regulates the orientation of stereocilia in the inner ear, the direction in
which fur grows, and convergent extension movements during gastrulation and neurulation\textsuperscript{1,2}.
The core PCP components are conserved in vertebrates, though there are several key differences
in the signaling pathway between Drosophila and vertebrates. For example, because of gene
duplication, there are multiple genes encoding homologs of core PCP components in vertebrates,
increasing the complexity of the vertebrate PCP pathway\textsuperscript{2}. In Drosophila, there are four Frizzled
homologs, and one of Vang, Dishevelled, and Prickle\textsuperscript{2}. In comparison, there are ten Frizzled
homologs, two Vangl, three Dishevelled, and two Prickle homologs in the mouse\textsuperscript{2}.

PCP signaling regulates directed cell migration and cell intercalation during tissue
remodeling\textsuperscript{1,2}. One example of a process requiring tissue remodeling in vertebrates is convergent
extension, a process that is not regulated by PCP in Drosophila. During convergent extension and
neural tube development, PCP signaling regulates cell migration, cell division, and changes cell
shape by narrowing cells in the medial/lateral axis (figure 4). Cell division and the intercalation
of cells across the midline of the body axis results in the lengthening of the embryo in the
anterior/posterior direction. Though PCP does not regulate convergent extension in Drosophila,
PCP signaling is required in the eye imaginal disc during the rotation of photoreceptors in the
ommatidia\textsuperscript{1,3,4}. One difference between cell movement in the imaginal disc and neurulation in
vertebrates is that cell adhesion in the imaginal disc is closely maintained and epithelial-like. In
contrast, mesodermal and neuroectodermal cells in the neural tube have mesenchymal character
during cell migration and cell adhesion is looser\textsuperscript{1}. Lamellipodia and filopodia are necessary for
directed cell movement during convergent extension, which are not required for ommatidial rotation\(^1\).

The asymmetric localization of the core PCP components have not been investigated extensively in vertebrates. However, it has been shown in zebrafish that Prickle localizes to the anterior membrane of neuroepithelial cells during neurulation\(^5\). In maternal-zygotic vangl2 (MZvangl2) mutants, Prickle localization is disrupted and becomes cytoplasmic\(^5\). In addition, Prickle localizes to the anterior while Dishevelled localizes to the posterior side of the cell during convergent extension movements in the zebrafish\(^21\). It has also been shown that Prickle2 and Vangl1 are localized anteriorly and Dishevelled2 and Dishevelled3 are localized posteriorly in the mouse node\(^22,23\). However, other evidence has shown that the asymmetric distribution of core PCP components is not entirely conserved between Drosophila and vertebrates. For example, Frizzled and Stbm/Vang localize to opposite ends of the wing cells in Drosophila\(^1,2\). In contrast, a study done using inner ear sensory hair cells showed that Frizzled3 and Frizzled6 localize with Vangl2 at the same side of the cell\(^24\). Another key difference between vertebrate and Drosophila PCP is that non-canonical Wnts such as Wnt5b and Wnt11 have been identified as Frizzled ligands whereas the ligand for Frizzled has not been identified in Drosophila\(^11\).

Similar to Drosophila, domineering nonautonomy can also occur in vertebrate PCP. Cell shape changes of wild-type cells can be influenced by neighbouring mutant cells during zebrafish gastrulation\(^2,25\). For example, on transplantation of wild-type cells into a trilobite/vangl2 mutant embryo, the mediolateral polarization of the wild-type cells can be inhibited\(^22,25\). In addition, transplantation of wild-type hair follicles into vangl2 mutant skin can alter the polarization of hair growth from wild-type cells\(^2\). Finally, PCP signaling plays a role in controlling the polarization of cilia in both zebrafish and mouse, but cilia are absent from tissues in Drosophila.

### 1.5 Regulation of cilia by PCP signaling

Cilia are cellular protrusions that regulate biochemical and physiological cellular processes by acting as antennae to sense cues from the external environment\(^26-28\). These cues initiate signaling pathways within the cilium, which are then relayed into the cell body. In particular, receptor proteins, ion channels, and signaling complexes are localized to cilia, which
Figure 4. Convergent extension. During convergent extension, cells elongate in the medial/lateral (M/L) axis and intercalate, resulting in the lengthening of the embryo in the anterior/posterior (A/P) direction.
act as centres for signaling pathways such as the Hedgehog signaling pathway\textsuperscript{26-30}. The ciliary axoneme is composed of an array of microtubules encapsulated by a specialized membrane compartment that is distinct from the plasma membrane\textsuperscript{31}. The microtubules in the majority of motile cilia are in the 9+2 configuration whereas the microtubules in the majority of nonmotile cilia are in the 9+0 configuration\textsuperscript{26}. Cilia in the 9+2 configuration have nine doublet microtubules surrounding a central pair of microtubules which is absent in cilia in the 9+0 configuration\textsuperscript{26}. The basal body is docked beneath the membrane and serves as a template for the growth of the ciliary axoneme. Since protein synthesis does not occur in the ciliary axoneme, proteins are trafficked into the cilium by intraflagellar transport (IFT) proteins\textsuperscript{28}. IFT proteins regulate the growth of cilia or “ciliogenesis.” Anterograde protein transport is mediated by kinesins, which associates with IFT complex B whereas retrograde protein transport is mediated by dyneins, which associates with IFT complex A\textsuperscript{28}. These complexes bind to cargo through protein-protein interactions. Ciliogenesis is initiated only when cells exit the cell cycle; conversely, ciliary reabsorption allows cells to enter the cell cycle\textsuperscript{28}.

The trans-Golgi network (TGN) is in close proximity to the basal body and has an important role in the transport of cargo to cilia. The intraflagellar transport protein IFT20 is a component of IFT complex B and is also localized to the trans-Golgi network\textsuperscript{28,32}. IFT20 is responsible for the transport of proteins from the TGN to the basal body; these proteins will initiate formation of the cilium. Knockdown of IFT20 proteins in human retinal pigment epithelial cells inhibits ciliogenesis though without disrupting the trans-Golgi network or IFT complex B\textsuperscript{28}. Protein transport into the cilia is tightly regulated. Septins act as diffusion barriers to regulate the entry of cytoplasmic proteins into the cilia, and maintain the signaling efficiency of the cilia\textsuperscript{31}. About 1000 different types of proteins are required at the base of cilia to maintain the normal function of cilia\textsuperscript{28}.

Numerous human diseases are caused by defects in ciliogenesis and ciliary function. Vertebrate models have been useful in studying ciliopathies since almost all vertebrate tissue have cilia that can play tissue-specific roles throughout embryonic development and adulthood. Because cilia are found in almost all types of tissues, diseases affecting cilia can have pleiotropic consequences\textsuperscript{26,28}. For example, the whip-like motion of flagella that is required for the movement of sperm requires the function of ciliary proteins; hence, one of the symptoms of ciliary disease is immotile sperm\textsuperscript{26,28,33}. Cilia that line the respiratory mucosa beat forward and
backward synchronously in order to transport mucus and clear the respiratory airway. Defective airway clearance results in respiratory disease, which often affects patients with defects in the ciliary axoneme\textsuperscript{34}. Retinal degeneration is also a cilia-related disorder. In retinal photoreceptors, a connecting cilium connects the inner segment to the outer segment of membrane stacks containing photopigment\textsuperscript{26,35}. The connecting cilium is required for the transport of proteins that assemble and maintain the outer segment\textsuperscript{26,35}. Cilia are the signaling centres for the Hedgehog pathway, which regulates limb digit morphogenesis. Ciliopathies can cause polydactyly by disrupting limb patterning in the embryo. Finally, ciliopathies are the basis of genetic diseases such as Joubert syndrome, Bardet-Biedl syndrome, and polycystic kidney disease\textsuperscript{36-38}.

One of the most important roles that cilia play during embryonic development is to initiate the establishment of left/right asymmetry. At embryonic day 7.5, rotating cilia in the node generate a leftward fluid flow in the mouse embryo, which results in the breaking of symmetry in the mouse embryo\textsuperscript{26}. Two different models have been proposed to explain how left/right asymmetry is generated by nodal flow. In the first model, nonmotile mechanosensory cilia at the periphery of the node sense the nodal flow that is generated by motile cilia in the centre of the node\textsuperscript{39}. The sensing of nodal flow by the mechanosensory cilia then generates downstream signaling events leading to the breaking of symmetry\textsuperscript{39}. In the second model, nodal flow results in the movement of a morphogen gradient across the node\textsuperscript{40}. This morphogen is most likely vesicles that contain Sonic Hedgehog ligand or retinoic acid\textsuperscript{40}. These vesicles are transported along by nodal flow until the vesicles are broken and the molecules are released\textsuperscript{40}. Individuals affected by ciliopathies can exhibit situ inversus, in which left/right body asymmetry is reversed\textsuperscript{26-28}.

PCP signaling regulates several aspects of cilia structure and function. For example, it has been suggested that Dishevelled regulates the positioning and orientation of cilia basal bodies\textsuperscript{41}. However, Dishevelled also participates in canonical Wnt signaling pathways, leaving open the question of whether PCP alone directly regulates basal body docking. Work in our lab using the zebrafish model has shown that Vangl2, a PCP-specific core component, regulates the proper orientation of cilia in Kupffer’s vesicle\textsuperscript{6}. The beating of motile cilia generates a leftward fluid flow to establish left/right asymmetry in the embryo. It has also been demonstrated in the mouse that PCP plays a role in the orientation of cilia in the node\textsuperscript{7}. This study used a double knockout mouse model to eliminate possible genetic redundancy between \textit{vangl1} and \textit{vangl2}\textsuperscript{7}. 
The double vangl\textsubscript{1}/vangl\textsubscript{2} mutants exhibited defects in heart looping and lung morphogenesis, phenotypes that are associated with PCP\textsuperscript{7}. Both studies in the zebrafish and mouse showed that Vangl was not required for ciliogenesis since normal cilia formation is unperturbed\textsuperscript{6,7}. Both studies also used bead injections to show that the rotating motion of cilia was randomized, compared to the normal clockwise flow in the mouse node and normal counterclockwise flow in the zebrafish Kupffer’s vesicle in wild-type animals\textsuperscript{6,7}. In addition, basal bodies were centrally docked in cells in the mouse node and in floorplate cells in the zebrafish neural tube\textsuperscript{6,7}. Lefty and Nodal were bilaterally expressed in the lateral plate mesoderm (LPM) instead of asymmetrically expressed on the left side of the LPM\textsuperscript{6,7}. Furthermore, in the zebrafish, cilia in the neural tube point posteriorly into the lumen, and the angle between the cilia and apical membrane is biased\textsuperscript{6}. However, in MZ\textsubscript{vangl2} mutants, the angle becomes randomized\textsuperscript{6}. Thus, evidence in both mice and zebrafish point towards the PCP signaling pathway as a mechanism that regulates ciliary orientation and motion.

1.6 Identifying novel vertebrate-specific PCP signaling components

Most of our knowledge of PCP signaling pathways is derived from genetic screens using Drosophila. The asymmetric subcellular localizations of the core PCP components and their molecular interactions were first described using Drosophila as a model. However, studies using both the mouse and zebrafish as a model have shown that there are key differences between Drosophila and vertebrate PCP, namely in the localizations and possible functions of the signaling components. Hence, PCP signaling is not entirely conserved between Drosophila and vertebrates, and vertebrate-specific mechanisms of PCP regulation may exist. For example, convergent extension is not controlled by PCP in Drosophila, and Drosophila do not have cilia. Therefore, identifying novel vertebrate-specific PCP signaling components may reveal mechanisms of PCP regulation that are not found in Drosophila.

Frizzled and Dishevelled are core PCP components that participate in both canonical and non-canonical Wnt signaling pathways. Therefore, it would be more useful to study Vangl2 to understand PCP-specific processes since Vangl2 is a PCP-specific core component that participates only in non-canonical Wnt signaling. It would also be necessary to characterize interacting partners of Vangl2 to further our understanding of how Vangl2 regulates cilia.
Figure 5. The membrane yeast two-hybrid screen can detect interactions between transmembrane proteins. (A) The protein of interest (bait) is fused to the C-terminal of ubiquitin in frame with the artificial transcription factor LexA-VP16. Interaction of the bait is tested against an array of different prey, which is fused to the N-terminal of ubiquitin. (B) If the bait and prey interact, this reconstitutes the ubiquitin, and the artificial transcription factor is cleaved by intracellular proteases. Translocation of the transcription factor into the nucleus activates the transcription of reporters which confers viability to the yeast on selective media.
Figure 6. Tmem14a contains three or four putative transmembrane domains. (A) The unspliced *tmem14a* mRNA transcript is 4.33 kb and contains 5 exons and four introns. (B) Zebrafish and human Tmem14a share 51% identity and 76% similarity. The protein has at least 3 transmembrane regions (the putative fourth transmembrane region is shown in grey). (C) The protein topology prediction for Tmem14a shows that there are very few amino acids on the cytosolic surface that could be used as an antigen to generate an antibody.
Figure 7. Possible protein topologies for Tmem14a based on the Tmem14a/Vangl1 interaction detected by MYTH screen. (A) Tmem14a could be a 3 pass (confirmation 1) or 4 pass (confirmation 2) transmembrane protein. However, in order for an interaction with Vangl1 to occur, Tmem14a would more likely be a 3 pass transmembrane protein. (B) The interaction between Tmem14a and Vangl1 could occur at the cell membrane, the trans-Golgi network, or ER. Note that it is possible for an interaction to occur if Vangl1 was at the cell membrane and Tmem14a was at the trans-Golgi network. In the MYTH screen, Tmem14a was tagged at the C-terminal and Vangl1 was tagged at the N-terminal.
function and PCP signaling since the function of Vangl2 is relatively uncharacterized.

Interaction studies that have been performed in Drosophila show that Vang can physically interact with both regulators of PCP (Diego, Prickle, Dishevelled, and Flamingo) and apical/basal polarity (Scribble). In Drosophila, Stbm interacts with Discs Large to promote membrane formation. In Drosophila, because all tissue lack cilia, the genetic screens that have been used to identify interactors of Stbm/Vang may not have been able to identify interactors that are involved in cilia growth and function.

Several physical interactors of Vangl2 have been identified in vertebrates. For example, Rack1 was identified in yeast two-hybrid screen for proteins that interact with a cytoplasmic portion of Vangl2. This screen used a cDNA library from embryonic mouse cochlear epithelia to identify interactors of Vangl2. Rack1, receptor for activated C kinase 1, is a scaffold protein that binds to activated PKC and is involved in targeting different receptors to the membrane. Knockdown of Rack1 disrupted convergent extension and oriented cell division during gastrulation, as well as the membrane localization of Vangl2. Vangl2 also interacts with Dact1, a protein that binds to Dishevelled and regulates Vangl2 activity at the primitive streak during mouse embryogenesis. In Dact1 mutants, dysregulation of Vangl2 activity causes disruption of E-cadherin localization in cells undergoing epithelial-to-mesenchymal transition. Bbs8 is a basal body protein that interacts with Vangl2 to establish left/right asymmetry in the zebrafish embryo. Knockdown of Bbs8 mRNA activity in a Vangl2 mutant background results in convergent extension defects, fused eyes, and a reduction in the number and length of cilia in Kupffer’s vesicle.

With respect to trafficking pathways, it is known from studies in the mouse that Ror2 and Vangl2 form a complex and that Ror2 plays a role in phosphorylating Vangl2. Phosphorylation of Vangl2 is necessary for proper trafficking of Vangl2 from the ER. This study proposed that a Wnt5a gradient is sensed by the Ror2-Vangl2 complex during limb bud development. Sensing of a Wnt5a gradient results in polarization of chondrocytes in the limb bud and elongation of the limb in the proximal/distal axis.

Sec24b is a component of the COPII complex that interacts with Vangl2 to selectively sort Vangl2 from the ER to the Golgi. Sec24b mouse mutants exhibit craniorachischisis and other PCP phenotypes such as omphalocele (intestinal tissue fail to fuse at the midline, and thus intestines and liver develop outside the abdomen), misoriented hair cells in the outer and inner
ear, defects in convergent extension, and failure in eyelid fusion. The Sec24b mutation was isolated from a forward genetic screen for mutations disrupting neural tube development. The allele harbors a nucleotide substitution and encodes for a truncated protein that fails to interact with other components of the COPII coat protein complex. COPII vesicles transport cargo from the ER to the Golgi and is the first step in the targeting of a large number of proteins to their proper subcellular localizations. Sec24b forms a complex with Sec23 in the cytosol and is then recruited to sites of active budding in the ER. Sec24b interacts with different cargo proteins in the ER to package these proteins in COPII vesicles. The Sec24b allele that was isolated encodes a truncated protein that cannot interact with Sec23, and thus is not recruited to cargo budding sites in the ER. This was demonstrated in co-immunoprecipitation experiments in which wild-type Sec24b protein was able to interact with Sec23 whereas the truncated version could not. Furthermore, the sec24b allele genetically interacted with vangl2. Using an in vitro vesicle budding assay, it was shown that Vangl2 was packaged into COPII vesicles when Sec23a/Sec24b complexes were added to the in vitro reaction. Furthermore, mutated versions of Vangl2 could not be packaged into COPII vesicles. Finally, the neural tube of Sec24b mutants showed a defect in the membrane localization of Vangl2. This study underlines the importance of trafficking pathways to target Vangl2 properly to the membrane for PCP signaling to occur. No other known trafficking proteins that interact with Vangl2 have been isolated and it is not known whether Vangl2 interacts with a Golgi-localized protein for proper sorting to the cell membrane.

An investigation into the interacting partners of Vangl2 may reveal other signaling components that also function specifically in PCP signaling pathways. In addition, it would be useful to characterize Vangl1, another member of the Vangl protein family that shares 60% identity and 74% similarity with Vangl2. Vangl1 has not been as well characterized as Vangl2, though it has been suggested in the mouse model that Vangl1 plays a similar role as Vangl2 in neural tube closure. Since Vangl2 is a transmembrane protein, using a mass spectrometry approach would be difficult to identify new potential interactors. Regular yeast two-hybrid screens are also not possible since the transmembrane protein is anchored to the membrane and cannot translocate into the nucleus to activate the reporter. Therefore, a Membrane Yeast Two-Hybrid (MYTH) screen was used in our lab to identify new interacting partners of Vangl1 and Vangl2. A MYTH screen utilizes a version of the protein of interest (bait) that is fused to the C-
terminal of ubiquitin in frame with the artificial transcription factor LexA-VP16 (figure 5). Interaction of the bait is tested against an array of different prey, which is fused to the N-terminal of ubiquitin. If the bait and prey interact, this reconstitutes the ubiquitin, and the artificial transcription factor is cleaved by intracellular proteases. Translocation of the transcription factor into the nucleus activates the transcription of a reporter which confers viability to the yeast.

Using this approach, an uncharacterized transmembrane protein, Tmem14a, was identified in the MYTH screen as potential interactor of Vangl1. Tmem14a is an uncharacterized, vertebrate-specific protein with unknown function and is evolutionarily conserved between species. Tmem14a orthologs have been identified in human, mouse, rabbit, chicken though no orthologs have been found in Drosophila. The human and zebrafish Tmem14a share 51% identity and 76% similarity (figure 6). In zebrafish, the genomic locus is located on chromosome 13 and spans 4.33 kb. Two different transcript variants can be processed, both containing 5 exons and encoding for a 99 amino acid protein (transcript IDs: ENDSART00000140423 and ENDSART00000143356). The Tmem14a protein is predicted to contain three or four transmembrane regions (figure 7) but no known functional domains, and is a putative member of the tetraspanin protein family. Tetraspanin proteins are involved in trafficking and are localized to the ER, Golgi, and various vesicles inside the cell. In humans, the tetraspanin protein Tmem216 has been linked to Meckel Gruber syndrome and other ciliopathy-related disorders.

1.7 The trans-Golgi network

The trans-Golgi network is a compartment of the Golgi where proteins and lipids are sorted into vesicles that are delivered to the apical or basolateral membrane, endosomes, and other compartmental sites in the cell. The Golgi is comprised of interlinking stacks shaped into reticular-like structures that are organized around the centrosome. Vesicles are transported along microtubule networks by motor proteins. In mammalian cells, dynein is a minus-end directed motor that forms a complex with dynactin and attaches Golgi membranes to microtubule networks in the cytoplasm. In addition to being the sorting centre for proteins, the Golgi is also the site where proteins are post-translationally modified by glycosylation, ubiquitylation, and phosphorylation. Polarized trafficking of Golgi vesicles is essential for establishing and
maintaining the characteristic boundaries between different membrane regions of the cell. For example, epithelial cells have an apical surface that faces the lumen and a basolateral surface that contacts neighbouring cells and the bottom layer of connective tissue; these surfaces are kept separate by tight junctions that exclude the different membrane compartments\textsuperscript{55}. Organization of the trans-Golgi networks into microdomains facilitates polarized trafficking of proteins and lipids\textsuperscript{56}. Thus, the trans-Golgi network plays an essential role in establishing and maintaining the physiological function of epithelial cells.

1.8 Tetraspanins regulate the trafficking and transport of proteins into cilia

The ciliary membrane is a specialized signaling compartment that is distinct from the plasma membrane, and therefore, trafficking of both cytoplasmic and membrane proteins into the cilia is tightly regulated\textsuperscript{57}. The transition zone is the region separating the ciliary membrane from the plasma membrane and includes the microtubule axoneme, the basal body, and Y-shaped protein structures that link the axoneme to the ciliary membrane\textsuperscript{57}. A recent study has demonstrated that a nuclear pore-like structure acts like a diffusion barrier to limit the types of cytoplasmic proteins that can enter the cilia\textsuperscript{57}. Similar mechanisms that regulate transport into the nucleus also regulate transport into cilia. For example, nuclear localization-like signals are required for ciliary entry\textsuperscript{57}. Ciliary entry is regulated by the nuclear transport factor importin-B2 and RanGTP/GDP gradient, two mechanisms that also regulate nuclear entry\textsuperscript{57}. Thus, the movement of proteins into the cilia is a tightly regulated process.

Studies have also demonstrated the emerging role of tetraspanins in the trafficking of proteins into cilia. These tetraspanins localize to the base of the cilia. For example, Tmem216 localizes to the base of cilia in mouse inner medullary collecting duct (IMCD3) and human retinal pigment epithelium (hRPE) cells\textsuperscript{52}. siRNA knockdown of Tmem216 in IMCD3 cells disrupted ciliogenesis and basal body docking at the apical surface\textsuperscript{52}. In addition, Tmem216 physically interacts with Tmem67 and forms a complex with RhoA and Dishevelled\textsuperscript{52}. On knockdown of Tmem216, RhoA and Dishevelled become hyperactivated, resulting in defects in basal body docking\textsuperscript{52}. A similar study was performed focusing on Tmem237, a component of a complex localized to the base of cilia that regulates diffusion of proteins into the transition
Ciliogenesis was disrupted in fibroblasts derived from individuals with mutations in Tmem237, who exhibit Joubert syndrome, and hyperactivation of RhoA and Dishevelled was also observed. Tmem216 and Tmem237 zebrafish morphants exhibit convergent extension phenotypes, which can be rescued by injections of human mRNA encoding Tmem216 or Tmem237.

Tmem231 and Tmem17 are two other tetraspanins that both localize to the transition zone and are required for the recruitment of the ciliopathy complex to the transition zone. The ciliopathy complex is a complex of proteins at the base of cilia that are involved in the initiation and maintenance of cilia. Nine proteins found in the ciliopathy complex include B9D2, TCTN1, TCTN2, MKS1, AHI1, CC2D2A, Tmem231, Tmem17, and KCTD10, and mutations in each have been linked to human genetic diseases caused by defects in the growth or maintenance of cilia. The ciliopathy complex acts as a diffusion barrier at the transition zone, preventing proteins from passing freely between the ciliary and plasma membrane. Using IMCD3 cells that express SSTR3-GFP, a GFP-tagged receptor that localizes to cilia, the authors demonstrated that siRNA knockdown of Tmem231 and Tmem17 in IMCD3 cells reduced the amount of SSTR3-GFP receptor localization in cilia. Using fluorescence recovery after photobleaching (FRAP), the authors showed that SSTR3-GFP diffused rapidly into cilia as a result of the absence of a diffusion barrier at the transition zone. Knockdown of Tmem231 also results in delayed ciliogenesis or short cilia, also as a result of proteins diffusing out of the cilia. Thus, Tmem231 and Tmem17 are membrane-bound at the transition zone to components of the ciliopathy complex and acts as a diffusion barrier for the efficient growth of cilia and to prevent receptor proteins in the cilia from diffusing back into the plasma membrane. Therefore, tetraspanins have a demonstrated role in regulating protein trafficking into cilia, which suggests a plausible link between Tmem14a and ciliary trafficking.

1.9 Objectives

The goal of this thesis is to determine whether Tmem14a is a novel component of the PCP signaling pathway. I focus on using confocal microscopy to analyze the subcellular localization of Tmem14a in the zebrafish embryo and on using morpholino knockdown approaches to examine whether knockdown of Tmem14a mRNA activity results in PCP-like
mutant phenotypes. These results show that GFP-tagged Tmem14a is localized to the trans-Golgi network and is associated with the base of cilia. Knockdown of Tmem14a mRNA activity in the zebrafish resulted in convergent extension defects, curved body axes, disorganized cilia in the neural tube, and disrupted the membrane localization of Vangl2. Finally, using HEK293T cells, I show that overexpression of Vangl2 results in Golgi scattering.
Chapter 2

Results

2 Results

2.1 Introduction

Planar cell polarity (also called the non-Canonical Wnt pathway) refers to the coordinated orientation, movement, or structure of cells within the plane of a tissue. Vangl1 and Vangl2 are PCP-specific members of a core set of proteins that regulate PCP signaling. In contrast, some core PCP components such as Frizzled and Dishevelled are also involved in a branch of Wnt signaling called the Canonical Wnt pathway and thus are not PCP-specific. Hence, discovering new characteristics of these proteins may not reveal new information about PCP-specific processes. Since Vangl2 is a PCP-specific core signaling component, I chose to focus this investigation on Vangl2. However, defining the cellular function of Vangl2 is difficult due to the relative paucity of interacting partners that have been discovered for Vangl2. With this in mind, a membrane yeast-two hybrid screen was performed by others in the lab that identified Tmem14a as a potential interactor of Vangl2.

In previous work from the lab, a MYTH screen was performed with the goal of discovering novel interactors of Vangl2. The screen used a human fetal brain cDNA library to generate the prey proteins, which were tagged on the C-terminus. Both human Vangl1 and Vangl2 were used as bait proteins in the screen and were tagged on the N-terminus. Vangl1 and Vangl2 are 60% identical at the amino acid level and Vangl1 can rescue the maternal-zygotic Vangl2 mutant phenotype, suggesting that Vangl1 shares some of the same functions as Vangl2. We therefore expected that most proteins identified in the MYTH screen would interact with both Vangl1 and Vangl2. However, there are some notable differences between Vangl1 and Vangl2. For example, in the zebrafish, Vangl2 is maternally deposited whereas Vangl1 is not expressed until the 15 somite stage. Their spatial expression patterns are also different: Vangl1 is widely expressed in the neuroectoderm, neural tube, and hatching gland whereas Vangl2 expression is restricted to the hindbrain. More importantly, morpholino
knockdown of Vangl1 does not result in a noticeable embryonic phenotype whereas knockdown of Vangl2 results in early convergent exension, neurulation, facial bronchiomotor and cilia defects\textsuperscript{5,6,59,60}. Thus, although the MYTH screen used both Vangl1 and Vangl2 as bait proteins, Vangl2 is more relevant to embryonic PCP. Therefore, follow-up analyses of novel proteins identified through the MYTH screen were performed in the context of Vangl2 interacting proteins.

Using Vangl1 as bait, the MYTH screen returned 90 hits of which 39 were unique hits\textsuperscript{8}. The most frequent hit was RNF41 (20 out of the 90 hits were RNF41) and the function of RNF41 is currently being investigated by a research associate in the lab. The second most frequent hit was Tmem14a (12 out of the 90 hits were Tmem14a)\textsuperscript{8}. When Vangl2 was used as bait, the screen returned 67 hits of which 56 were unique hits\textsuperscript{8}. Again, RNF41 was the most frequent hit (7 out of the 67 hits were RNF41)\textsuperscript{8}. Although Tmem14a was not detected, this is possibly because the screen was not saturating\textsuperscript{8}. Indeed, other components of the PCP signaling pathway that are known to interact with Vangl2, such as Prickle, were not detected\textsuperscript{8}.

Tmem14a is an integral membrane protein and is predicted to contain 3 or 4 transmembrane domains based on ALIGN Query and TMHMM prediction software (figure 6). However, for the MYTH screen to identify Vangl1 as an interacting protein, the C-terminus of Tmem14a must be cytosolic, favoring the 3 pass transmembrane topology (figure 7). There are no signal sequences in the protein sequence for Tmem14a that can suggest a specific subcellular localization\textsuperscript{61}. There are a total of five members in the Tmem14 protein family, all of which are short transmembrane proteins with a length of \textasciitilde 100 amino acids. Tmem14b, Tmem14d, and Tmem14e are entirely uncharacterized proteins. Both Tmem14a and Tmem14c are relatively uncharacterized, and have been referenced in the literature only once\textsuperscript{61,62}. Tmem14c was identified as a putative mitochondrial transporter from a computational screen identifying proteins involved in heme biosynthesis\textsuperscript{62}. The computational screen utilized microarray data sets and algorithms to search for genes that were coexpressed with heme biosynthesis genes\textsuperscript{62}. Knockdown of Tmem14c in zebrafish embryos resulted in severe anemia\textsuperscript{62}. However, the authors did not confirm through subcellular co-localization studies that Tmem14c was indeed localized to the mitochondria.

Tmem14a has been characterized by others as an inhibitor of apoptosis\textsuperscript{61}. Using cell culture systems, Tmem14a was found to be localized to the mitochondria and ER\textsuperscript{61}. The study
showed that Tmem14a inhibits apoptosis by suppressing Bax, a member of the proapoptotic Bcl-2 protein family\textsuperscript{61}. Proapoptotic events such as the release of cytochrome C and caspase-3 activation were blocked in U87MG cells when Tmem14a was overexpressed\textsuperscript{61}. Conversely, apoptosis was increased when Tmem14a expression was suppressed using shRNA\textsuperscript{61}. The implications of this study with respect to the results generated in this thesis will be further discussed later in this chapter.

The overall goal of this project was to characterize Tmem14a as interactor of Vangl2 and to investigate whether Tmem14a has a role in trafficking Vangl2 from the trans-Golgi network to the membrane. Here I use fluorescently-tagged proteins and morpholino knockdown as approaches to characterize Tmem14a and to investigate the potential function of Tmem14a as a novel member of the PCP signaling pathway. Tmem14a is localized to the trans-Golgi network in the zebrafish embryo. Tmem14a morphants display convergent extension defects, which indicate a disruption in PCP signaling, as well as curved body axes, which indicate a defect in ciliogenesis and/or apicobasal polarity. An analysis of cilia in the neural tube showed that cilia were severely disorganized. GFP-Vangl2 localization was disrupted in Tmem14a morphants. Finally, in vitro studies using HEK293T cells showed that overexpression of GFP-Vangl2 resulted in Golgi scattering, which is rescued upon co-expression of Tmem14a-myc.

2.2 Tmem14a and Vangl2 can physically interact

To confirm that zebrafish Vangl2 and Tmem14a physically interact, I performed co-immunoprecipitation experiments in HEK293T cells. Tmem14a-myc and GFP-Vangl2 expression constructs were generated using Gateway technology. HEK293T cells were transfected with GFP-Vangl2, Tmem14a-myc, or both constructs and an anti-myc antibody was used to immunoprecipitate Tmem14a-myc. The membrane was blotted with both anti-myc and anti-GFP antibodies. Tmem14a-myc (apparent molecular weight 30 kDa) was pulled down from the lysate when cells were transfected with Tmem14a-myc alone (figure 8). As a negative control, HEK293T cells were transfected with GFP-Vangl2 alone. Immunoprecipitation using the anti-myc antibody did not pull down GFP-Vangl2 (figure 8), demonstrating that there was no residual binding of GFP-Vangl2 to the beads used in the immunoprecipitation. Finally, Tmem14a-myc and GFP-Vangl2 (87 kDa) were co-immunoprecipitated when HEK293T cells
were transfected with both constructs (figure 8). These results confirm that GFP-Vangl2 and Tmem14a-myc can physically interact.

2.3 \textit{tmem14a} mRNA is expressed throughout development

The zebrafish \textit{tmem14a} gene is located on chromosome 13. The length of the transcript is 4 kb, and includes five exons and four introns (figure 6). The length of the fully spliced mRNA coding sequence is 300 bp, which encodes a very small protein composed of 99 amino acids, and is predicted to contain three or four transmembrane domains (figure 6). To begin characterizing \textit{tmem14a}, the mRNA expression of \textit{tmem14a} was examined in total RNA isolated from whole embryos at different stages of development using RT-PCR. The RT-PCR results showed that \textit{tmem14a} mRNA is expressed during all stages of embryonic development, from the 4 cell stage to 48 hours post-fertilization (hpf) (figure 9). Since zygotic transcription does not initiate until the 1000 cell stage, these results also showed that \textit{tmem14a} mRNA is maternally deposited. Sequencing of the cDNA confirmed that \textit{tmem14a} was amplified during the RT-PCR reaction. In situ hybridization was also used to visualize the spatiotemporal expression of \textit{tmem14a}. However, these attempts failed to detect \textit{tmem14a} mRNA expression, possibly because the expression level of \textit{tmem14a} is very low.

2.4 GFP-tagged Tmem14a is localized to the Golgi

The protein topology prediction for Tmem14a shows that there are very few amino acids on the cytosolic surface that could be used as an antigen to generate an antibody (figure 6). Therefore, it would be difficult to raise an antibody against Tmem14a. To determine its subcellular localization in the zebrafish neural tube, Tmem14a was tagged with GFP or mCherry. To examine the localization of Tmem14a protein during different stages of embryonic development, \textit{tmem14a-GFP} or \textit{tmem14a-mCherry} mRNA were injected into zebrafish embryos at the one-cell stage and confocal microscopy was used to visualize the protein in the neural tube at the 12 to 16 somite stage.

Previously published papers predict that Tmem14a and Tmem14c are localized to mitochondria\textsuperscript{61,62}. Therefore, co-localization of Tmem14a-mCherry with the mitochondria was first examined. A mitochondria-GFP construct adapted from Kim et al. (2008) was used to label
Figure 8. Tmem14a-myc and GFP-Vangl2 can physically interact. HEK293T cells were transfected with Tmem14a-myc construct (lane 1 and 4), GFP-Vangl2 construct (lane 2 and 5) or both (lane 3 and 6). An anti-myc antibody was used to co-immunoprecipitate Tmem14a-myc. The membrane was blotted with both anti-myc and anti-GFP antibodies. For input lanes, 20 µg of protein was used. (The MW of GFP-Vangl2 is 87 kDa, the apparent MW of Tmem14a-myc is 30 kDa). The extra bands in lane 6 may be due to degradation of GFP-Vangl2.
Figure 9. *tmem14a* mRNA is expressed throughout development. Total RNA was extracted from staged embryos and RT-PCR was used to detect *tmem14a* mRNA. The presence of *tmem14a* mRNA during the 8 cell stage indicates that *tmem14a* mRNA is maternally expressed.
the mitochondria. This construct encodes for a GFP that is tagged to the mitochondrial localization signal sequence from human cytochrome c oxidase. \textit{tmem14a-mCherry} mRNA and \textit{mitochondria-GFP} mRNA were injected into zebrafish embryos at the one-cell stage and confocal microscopy was used to visualize the proteins in the neural tube at the 16 somite stage. In both cells of the enveloping layer and the neural tube of the 16 somite stage embryo, mitochondria-GFP were dispersed throughout the cytoplasm, and did not co-localize with Tmem14a-mCherry (figure 10). Mitochondria-GFP was excluded in some areas of Tmem14a-mCherry labeling, indicating that Tmem14a-mCherry labeled structures do not co-localize with mitochondria (figure 10). Since Tmem14a-mCherry did not co-localize with mitochondria-GFP, co-localization with the trans-Golgi network was next examined. A DsRed-GalT reporter (Clontech) was used to label the trans-Golgi network. This reporter uses the sequence for a galactotransferase (GalT) that is targeted to the trans-Golgi network. \textit{tmem14a-GFP} mRNA and \textit{DsRed-GalT} mRNA were injected into zebrafish embryos at the one-cell stage and confocal microscopy was used to visualize the protein in the neural tube at the 12 somite stage. The DsRed-GalT marker co-localized with Tmem14a-GFP in reticular structures that extended from the perinuclear region to beneath the apical membrane (figure 11). Tmem14a-GFP also localized to perinuclear, ER regions (figure 11).

To verify that the Golgi localization of Tmem14a was not an artifact of GFP-tagging the protein on the C-terminus, an N-terminally GFP-tagged construct (GFP-Tmem14a) was generated and its localization in neuroepithelial cells was examined during the 16 somite stage. Similar to Tmem14a-GFP, GFP-Tmem14a labelled apically directed, reticular-like structures that extended from the perinuclear region to beneath the apical membrane (figure 11). Perinuclear, ER staining was also observed (figure 11). Thus, the similar localization of both Tmem14a-GFP and GFP-Tmem14a supports that GFP-tagged Tmem14a is localized to the Golgi and ER, though co-localization with an ER marker is needed to absolutely confirm this.

Mitochondria-GFP and DsRed-GalT are both well established markers that label the mitochondria and trans-Golgi network, respectively. Tmem14a-mCherry does not co-localize with mitochondria-GFP though Tmem14a-GFP does co-localize with DsRed-GalT. It can be speculated that Tmem14a is involved in the trafficking of core PCP components such as Vangl2 to the membrane. Or, Tmem14a may play a role in structuring the Golgi such that it extends to the apical surface. It can also be speculated that Tmem14a is involved in the tethering of the
Figure 10. Tmem14a-mCherry does not co-localize with mitochondria-GFP. Embryos were injected at the one cell stage with mRNA encoding Tmem14a-mCherry and Mitochondria-GFP and the enveloping layer (EVL; top panels) and neural tube (NT; bottom panels) were imaged at the 16 somite stage. Mitochondria-GFP are dispersed throughout the cytoplasm and are excluded in some areas of Tmem14a-mCherry labeling (arrows), indicating that Tmem14a-mCherry does not localize to the mitochondria. Photos courtesy of Dr. Brian Ciruna.
Figure 11. GFP-tagged Tmem14a co-localizes with DsRed-GalT, a marker of the trans-Golgi network. In top panels, GFP was tagged at the C-terminus of Tmem14a. In bottom panels, GFP was tagged at the N-terminus. Both versions of Tmem14a co-localize with DsRed-GalT. The DsRed-GalT marker co-localizes with GFP-tagged Tmem14a in reticular structures that extend from the perinuclear region to beneath the apical membrane (asterisks). GFP-tagged Tmem14a also localizes to perinuclear ER regions (arrows). mRNA was injected at the one cell stage and confocal images of the zebrafish neural tube were taken at the 12 to 16 somite stage. Anterior is up. Scale bars, 10 µm. Photos in top panel are courtesy of Dr. Brian Ciruna.
trans-Golgi network to structures in the cytosol, such as cilia basal bodies, or that Tmem14a is involved in the trafficking of Golgi vesicles to cilia.

2.5 Tmem14a is associated with the base of cilia

Several tetraspanin proteins such as Tmem216, Tmem237, Tmem231, and Tmem17 are associated with the base of cilia. In addition, the extended structure of the trans-Golgi network and its co-localization with Tmem14a-GFP suggested that the trans-Golgi network and Tmem14a-GFP may be associated with the base of cilia. To determine if the Tmem14a extension terminated at the cilia, the association of Tmem14a-mCherry with cilia was examined more closely. To visualize cilia, cells were labeled with Arl13b-GFP and Tmem14a-mCherry. Arl13b-GFP is an Arf/Arl GTPase that localizes to the ciliary axoneme. Labeled cells were transplanted into an unlabelled wild-type host and the neural tube was imaged using confocal microscopy. These transplants show that Tmem14a-mCherry encircles and extends to the base of the cilia (figure 12). Thus, the close association of Tmem14a-mCherry with cilia suggests that Tmem14a may have a role in regulating cilia. For example, Tmem14a may be involved in the trafficking of Golgi vesicles to cilia. Or, since the trans-Golgi network extends towards the base of cilia, Tmem14a may be involved in tethering the trans-Golgi network to cilia by interacting with a component of the basal body. Furthermore, Tmem14a may have a role in determining the tilt of cilia by regulating the positioning of cilia growth on the apical surface.

2.6 tmem14a morphants display curved body axes

To determine the function of Tmem14a, I examined the effect of knocking down Tmem14a activity. Translation blocking morpholinos (ATG-MO) bind to the ATG start codon on the mRNA, and this prevents the ribosome from translating the mRNA through steric hindrance. Translation-blocking morpholino was injected into one-cell stage embryos and their phenotypes were monitored at 24 hpf and 48 hpf. The morpholino was titrated and 4 ng of morpholino was found to be the optimal amount of morpholino that resulted in an observable phenotype with the least toxicity. At 4 ng of morpholino, 24 hpf embryos showed a range of phenotypes (figure 13; n=312 embryos from 26 individual experiments). For example, embryos showed a shorter and curved body axis with varying degrees of severity at which the length and
Figure 12. Tmem14a is associated with the base of cilia. Embryos were labeled with Tmem14a-mCherry and Arl13b-GFP to mark cilia. Cells were then transplanted into an unlabelled wild-type host and the neural tube was imaged using confocal microscopy. Structures labeled by Tmem14a-mCherry (asterisks) extend to the base of cilia. Tmem14a-mCherry also encircles the base of the cilia (arrows). Photos courtesy of Dr. Brian Ciruna.
Figure 13. *tmem14a* morphants injected with 4 ng of Tmem14a ATG-MO show curved body axes reminiscent of apical/basal polarity and ciliogenesis mutants. Panels on the left show embryos photographed at 24 hpf. Panels on the right show embryos photographed at 48 hpf. In comparison to wild-type (WT) embryos, embryos injected with 4 ng of Tmem14a ATG-MO (MO) show a curved body axis. At 24 hpf, embryos showed a shorter and curved body axis and there is a range in the degree of body curvature (two different embryos at 24 hpf are shown). The curved body axis persists at 48 hpf.
the degree of curvature was affected, with some embryos showing tails curved at the tip, or curved along the entire body axis. At 48 hpf, embryos displayed a curved body axis, a characteristic of apical/basal polarity mutants\textsuperscript{66,67} and cilia mutants\textsuperscript{68,69} (figure 13). A titration curve was performed using 2 ng, 3 ng, 3.5 ng and 4 ng of morpholino and resulted in a morphant phenotype severity that was concentration-dependent (figure 14A). Splice morpholinos were used to further confirm these phenotypes. In contrast to translation blocking morpholinos, the splice morpholino binds to the tmem14a mRNA at the junction between the second exon and the second intron and prevents splicing of the mRNA transcript (figure 15A). This results in either read through of the mRNA transcript and nonsense mediated decay, as the intron will contain cryptic termination codons that terminate protein translation; the synthesis of a truncated protein; or the generation of an alternatively spliced mRNA (figure 15A). Titrating an increasing amount of morpholino injected resulted in more severe phenotypes. For example, at 2 ng of Tmem14a splice-blocking morpholino, embryos exhibited tails that were bent at the tip, whereas 6 ng of Tmem14a morpholino resulted in more severe convergent extension defects and curved body axes (figure 15B).

To verify that the translation-blocking morpholino specifically targets and blocks tmem14a mRNA from being translated, tmem14a ATG morphants were rescued by co-injecting untagged, non-targetable tmem14a mRNA with 4 ng of Tmem14a morpholino. The non-targetable tmem14a mRNA is a version of the mRNA in which the first three codons after the ATG start codon are replaced with genetically redundant codons such that the ATG morpholino will not bind to the mRNA, though the mRNA will encode the same protein sequence. The non-targetable mRNA was generated using PCR in which the sequence of the PCR primers contains codons that are genetically redundant to the actual nucleotide sequence of the tmem14a mRNA. This method results in the incorporation of different nucleotides that will not be bound by the ATG morpholino.

First, titration of 100 pg, 200 pg, and 300 pg of the tmem14a mRNA into wild-type embryos confirmed that this did not result in an overexpression phenotype (figure 14A, n=20 embryos for each amount, 2 separate experiments). Tmem14a morphants could be rescued with 57% success at 100 pg of untagged tmem14a mRNA, and with 80% success at 200 to 300 pg of untagged tmem14a mRNA (figure 14B). These results confirmed that the translation-blocking morpholino specifically targets tmem14a mRNA since injecting in tmem14a mRNA after
Figure 14. Analysis of the *tmem14a* morphant phenotype.
(A) Embryos were injected with Tmem14a ATG-MO and were classified as normal, mild, or severe depending on the degree of body curvature.
(B) The Tmem14a morphant phenotype is concentration dependent. Embryos were injected with 2 ng, 3 ng, 3.5 ng, or 4 ng of morpholino at the one cell stage and their phenotypes were scored as normal, mild, or severe at the 48 hpf stage. The graph shows the percentage of each phenotype for each amount of morpholino injected. The number of embryos and the number of trials for each amount of morpholino injected are also shown below the graph.
(C) Tmem14a morphants can be rescued with *tmem14a* mRNA. Embryos were co-injected with 4 ng of morpholino and *tmem14a* mRNA. *tmem14a* mRNA were titrated at 0 pg, 100 pg, 200 pg, and 300 pg. Their phenotypes were scored as normal, mild, or severe at the 48 hpf stage. The graph shows the percentage of each phenotype for each amount of mRNA titrated.
Figure 15. *tmem14a* splice morphants display convergent extension defects and curved body axes similar to Tmem14a translation-blocking morphants. (A) The splice morpholino binds to the junction between exon 2 and intron 2. A truncated protein could be generated, as there is a stop codon in intron 2. An antibody would be needed in order to detect whether there is a truncated protein. Or, nonsense mediated decay could result. In this case, quantitative PCR would be needed to confirm that there is a reduction in mRNA levels. Finally, alternative splicing could occur in which exon 1 is spliced to exon 3. PCR would be needed to detect the presence of an alternatively spliced mRNA transcript. (B) Embryos were injected with 2 ng, 4 ng, and 6 ng of splice morpholino at the one cell stage and photographed at 24 hpf. The body curvature is more severe with each increase in the amount of splice morpholino injected.
knocking down the endogenous mRNA was able to restore the wild-type phenotype.

2.7 *tmem14a* morphants display convergent extension defects

During embryonic development, convergent extension results in the shortening of the embryo in the medial lateral axis and lengthening in the anterior posterior axis. A characteristic of PCP mutants such as MZvangl2 is a defect in convergent extension, which results in a shorter and broader embryo\(^5\). To analyze whether Tmem14a morphants show a defect in convergent extension, embryos were injected with 4 ng of translation blocking morpholino at the one cell stage and fixed at the 11 somite stage. Somites were labelled with *krox20/myoD* in situ probes (figure 16). I chose to analyze convergent extension using 4 ng of Tmem14a morpholino since this concentration of morpholino results in a morphant phenotype (a curved body axis in 48 hpf embryos) with the least toxicity. Morphants were stained for *krox20/myoD* at the 11 somite stage. Tmem14a show a slightly wider and shorter body axis compared to wild type (figure 16). Thus, *tmem14a* morphants have a convergent extension defect, though this defect is not as severe as MZvangl2 mutants. The length of the notochord and the width of the fifth rhombomere of each embryo was measured and the measurements were graphed for wild type and morpholino-injected embryos. The average length of the notochord is significantly shorter, and the average width of the fifth rhombomere is significantly longer, for morpholino-injected embryos compared to wild type (figure 16). Therefore, these results show that Tmem14a morphants exhibit a defect in convergent extension and PCP signaling, consistent with the interaction of Tmem14a with Vangl2.

2.8 Cells accumulate ectopically in the neural tube of *tmem14a* morphants

PCP signaling is required for the convergence of the neural plate during neural tube morphogenesis and for the re-integration of cells into the neuroepithelium after cell division\(^5\). A characteristic of PCP mutants such as MZvangl2 embryos is a broadened floorplate and an accumulation of cells within the neural tube\(^5\). This is because neuroepithelial cells fail to re-integrate into the epithelium after cell division\(^5\). Neural plate convergence was examined in
Figure 16. *tmem14a* morphants display defects in convergent extension. (A) The distance between the head and tail of the embryo is longer for *tmem14a* morphants compared to wild-type. Embryos were injected with 4 ng of translation-blocking morpholino at the one-cell stage and photographed at the 11 somite stage. (B) *Krox20/myoD* in situ hybridization of *tmem14a* morphants at the 11 somite stage. Morphant embryos are shorter and wider compared to wild-type controls. (C) The length of the notochord and the width of the fifth rhombomere were measured. The average length of the notochord is significantly shorter (p=0.0005) and the average width of the fifth rhombomere is significantly wider (p=0.0377) for *tmem14a* morphants compared to wild-type.
tmem14a morphants by labeling cell membranes with membrane-RFP. Embryos were co-injected with Tmem14a ATG morpholino and membrane-RFP RNA and the neural tube was imaged at 24 hpf. Similar to MZvangl2 embryos, tmem14a morphants showed an ectopic cell accumulation of neuroepithelial cells in the neural tube at the 24 hpf stage as well as a broader neural tube (figure 17). This further indicates that Tmem14a morphants have a defect in convergent extension.

2.9 Cilia form in tmem14a morphants, but show severe disorganization

The curved body axes displayed by tmem14a morphants suggest that there may be defects in cilia structure or function. In addition, the interaction of Tmem14a with Vangl2, which was shown to regulate both the orientation and localization of cilia in both mice\(^7\) and zebrafish\(^6\), suggests the possibility that Tmem14a co-operates with Vangl2 to regulate cilia. The association of Tmem14a with the trans-Golgi network and the extension of the trans-Golgi network to the base of the cilia suggest several potential roles for Tmem14a in regulating trafficking to the cilia or in structuring the trans-Golgi network. Therefore, cilia were examined to determine whether they formed normally in tmem14a morphants. Arl13b-GFP was used to visualize cilia in embryos at the 24 hr stage, during which the neural tube is lumenized. In wild type embryos (n=5), cilia line the midline of the neural tube and are positioned on the surface of the apical cell membrane such that they point in towards the lumen in the posterior direction (figure 17). Primary motile cilia are localized posteriorly and tilted in the posterior direction in floorplate cells, and the beating of cilia results in a fan-like appearance\(^6\). Cilia are present in tmem14a morphants (n=11), but are severely disorganized (figure 18). In more severe morphants (n=2), cells accumulated in the lumen of the neural tube, and length of the cilia appeared to be smaller (figure 18). Thus, preliminary data suggest that knockdown of Tmem14a appears to affect cilia structure, though it would be necessary to quantify in greater detail the motility, size, and structure of the cilia using electron microscopy and video microscopy.

2.10 The membrane localization of Vangl2 is disrupted in tmem14a morphants
Figure 17. Cells accumulate ectopically in the neural tube of *tmem14a* morphants. Embryos injected with 4 ng of morpholino and 15 pg of membrane-RFP show a broader neural tube (right) compared to wild-type (left). Morphant embryos show an ectopic accumulation of cells in the neural tube (arrow). Embryos were injected at the one-cell stage and the neural tube was imaged from the dorsal side at 24 hpf. Images are shown in grey scale. Anterior is to the left. Scale bars, 10 μm.
Figure 18. Cilia form in *tmem14a* morphants, but show severe disorganization. Embryos were injected with Arl13b-GFP and membrane-RFP at the one cell stage and the neural tube was imaged at 24 hpf. In wild-type embryos (n=5), cilia point towards the neural tube lumen in the posterior direction. Cilia are present in *tmem14a* morphants (n=11) but are severely disorganized, similar to MZvangl2 mutants. In more severe morphants (n=2), cells accumulated in the lumen of the neural tube, and length and width of the cilia appeared to be smaller. Anterior is up. Scale bars, 10 µm.
Figure 19. The membrane localization of GFP-Vangl2 is disrupted in *tmem14a* morphants. One cell stage embryos were co-injected with 4 ng of Tmem14a ATG morpholino and 100 pg of *gfp-vangl2* mRNA. The neural tube was imaged from the dorsal side at the 16 somite stage. In wild-type embryos, GFP-Vangl2 localizes to the membrane (n=9). *tmem14a* morphants showed reduced membrane localization and increased cytoplasmic localization of GFP-Vangl2 (n=4). In some cases, GFP-Vangl2 was completely cytoplasmic with no apparent membrane localization (n=2). Scale bars, 10 μm. Anterior is to the left.
Because asymmetries in the subcellular localization of core PCP components is a major theme in the regulation of PCP signaling, the localization of Vangl2 was examined in \textit{tmem14a} morphants. The Golgi localization of Tmem14a-GFP and the potential interaction between Tmem14a and Vangl2 suggests that Tmem14a may be involved in the trafficking of Vangl2. To test this possibility, the effect of knocking down Tmem14a activity on the membrane localization of GFP-Vangl2 was examined. One cell-stage embryos were co-injected with 4 ng of Tmem14a ATG morpholino and 100 pg of \textit{GFP-vangl2} mRNA, a concentration that does not result in an observable phenotype when injected alone, and the neural tube was imaged at the 16 somite stage. In wild-type embryos, GFP-Vangl2 localizes to the membrane (n=9) though no specific polarity (apical/basal or anterior/posterior) was observed for this localization (figure 19). \textit{tmem14a} morphants showed reduced membrane localization and increased cytoplasmic localization of GFP-Vangl2 (n=4), and in some cases, GFP-Vangl2 was completely cytoplasmic with no apparent membrane localization (n=2; figure 19).

Of interest, in one morphant embryo showing strong cytoplasmic GFP-Vangl2, perinuclear GFP-Vangl2 puncta and the localization of GFP-Vangl2 in reticular-like structures were detected (figure 20), which may suggest that knockdown of Tmem14a activity results in the trapping of GFP-Vangl2 in the ER or Golgi. However, this remains to be examined more closely by co-labeling the ER or Golgi and quantifying the co-localization of GFP-Vangl2 with the ER or Golgi.

To quantify the loss of GFP-Vangl2 localization in \textit{tmem14a} morphant embryos, the correlation between GFP-Vangl2 and membrane-RFP intensities was measured across four lines for three \(z\) stacks for each embryo and the mean correlation coefficient was calculated for six embryos. This analysis showed that the mean correlation coefficient for morpholino-injected embryos is significantly less compared to wild-type (figure 21). Therefore, the membrane localization of GFP-Vangl2 is disrupted upon knockdown of Tmem14a activity.

Cell transplant experiments were performed to determine whether Tmem14a has a cell autonomous role in the membrane localization of GFP-Vangl2. One cell stage embryos were co-injected with Tmem14a ATG-MO and GFP-Vangl2 and transplanted donor cells into a wild-embryo. In a WT\(\rightarrow\)WT controls, GFP-Vangl2 is membrane localized (figure 22). However, in a MO\(\rightarrow\)WT embryo, the localization of GFP-Vangl2 is still disrupted (figure 22), demonstrating
Figure 20. GFP-Vangl2 is potentially trapped in the Golgi or ER in *tmem14a* morphant embryos. In one morphant embryo showing strong cytoplasmic GFP-Vangl2, perinuclear GFP-Vangl2 puncta and the localization of GFP-Vangl2 in reticular-like structures (arrows) were detected. Image shows a single neuroepithelial cell in the neural tube of the morphant embryo.
Figure 21. The colocalization coefficient for GFP-Vangl2 and membrane-RFP is reduced for morphant versus wild-type embryos.

Fluorescence intensity profiles across a single wild-type cell (A) and a single tmem14a morphant cell (B) expressing GFP-Vangl2 and membrane-RFP. (A) In a wild-type cell, the peak intensities of GFP-Vangl2 and membrane-RFP overlap, indicating that GFP-Vangl2 co-localizes with membrane-RFP. (B) In a Tmem14a MO cell, membrane-RFP peak intensities coincide with reduced GFP-Vangl2 intensities. GFP-Vangl2 intensity is increased inside the cell, indicating that there is an increased expression of GFP-Vangl2 in the cytoplasm compared to the membrane.

The mean correlation coefficient is significantly reduced for tmem14a morphant embryos compared to wild-type embryos (p=<0.0005). To calculate the mean correlation coefficient, the correlation between GFP-Vangl2 and membrane-RFP fluorescence intensities was measured across four lines for three z stacks for each embryo and the mean correlation coefficient was calculated for six embryos.
Figure 22. Chimeric analyses show that Tmem14a plays a cell autonomous role in the membrane localization of GFP-Vangl2. Embryos were injected at the one cell stage with GFP-Vangl2 and membrane-RFP, with or without Tmem14a ATG-MO, and donor cells were transplanted into an unlabelled wild-type host at the early gastrulation (sphere) stage. The neural tube was imaged at the 16 somite stage. Anterior is to the left. Scale bars, 10 µm.
that Tmem14a plays a cell autonomous role in the membrane localization of GFP-Vangl2 since the wild-type environment does not rescue GFP-Vangl2 localization.

2.11 Overexpression of Tmem14a-GFP appears diffuse in HEK293T cells

It has been reported that Tmem14a is localized to the mitochondria\(^1\). In contrast, the data shown in this study demonstrate that GFP- and mCherry-tagged versions of Tmem14a are localized to the trans-Golgi network. To present further evidence that Tmem14a is localized to the trans-Golgi network, the localization of GFP-tagged Tmem14a was observed in HEK293T cells. The morphology of the trans-Golgi network was first examined by transfecting the DsRed-GalT construct into HEK293T cells; this showed that the TGN is condensed around the nucleus (figure 23A). Next, a construct expressing Tmem14a-GFP was transfected into HEK293T cells and the protein was observed using confocal microscopy. In contrast to the condensed DsRed-GalT localization, Tmem14a-GFP appears to be diffusely localized in the cytoplasm as well as in the perinuclear region (figure 23B). Rounded structures that may be Golgi vesicles were also observed (figure 23B) though it would be necessary to perform co-labeling using both Tmem14a-GFP and DsRed-GalT to confirm this.

These results suggest possible roles for Tmem14a in regulating Golgi structure. For example, Tmem14a may have a role in structuring the Golgi so that it is polarized towards one side of the cell since the TGN is normally condensed around the nucleus. The Golgi may be polarized towards a specific structure in the cell, such as the basal body of cilia, and Tmem14a may have a role in tethering the trans-Golgi network to cilia. Furthermore, it is possible that Tmem14a has a role in regulating the tilt of the cilia by determining where on the apical surface the cilia will form. Tmem14a may be responsible for the trafficking of Golgi vesicles towards the apical surface to initiate ciliogenesis.

2.12 Overexpression of GFP-Vangl2 in HEK293T cells causes Golgi scattering
Figure 23. In contrast to DsRed-GalT localization, overexpression of Tmem14a-GFP appears diffuse in HEK293T cells. (A) HEK293T cells were transfected with DsRed-GalT constructs. Under normal conditions, the trans-Golgi network is condensed around the nucleus. (B) HEK293T cells were transfected with Tmem14a-GFP constructs. The presence of rounded structures that may be Golgi vesicles was also detected (arrows).
Overexpression of Vangl2 in zebrafish embryos causes a convergent extension phenotype. To determine whether there is an effect on Golgi morphology after overexpression of Vangl2, HEK293T cells were used to overexpress GFP-Vangl2 and DsRed-GalT. GFP-Vangl2 and DsRed-GalT constructs were transfected into HEK293T cells and live cell imaging was used to examine the morphology of the trans-Golgi network. GFP-Vangl2 is targeted to the membrane of HEK293T cells (figure 24). However, the morphology of the Golgi, labeled by DsRed-GalT, appeared scattered when GFP-Vangl2 is co-expressed (figure 24). Normally, the Golgi is condensed around the nucleus in the absence of GFP-Vangl2 (figure 23A). Therefore, it is possible that GFP-Vangl2 has a role in shaping the morphology of the trans-Golgi network.

Next, the effect of co-expressing Tmem14a-myc and GFP-Vangl2 on Golgi morphology was observed. When Tmem14a-myc and GFP-Vangl2 constructs are co-expressed in HEK293T cells, this rescues the Golgi morphology and restores normal Golgi condensation around the nucleus (figure 24). Thus, Tmem14a-myc rescues the Golgi scattering that is caused by GPF-Vangl2 overexpression, possibly by interacting with and trapping GFP-Vangl2 in the Golgi and preventing it from signaling from the membrane. Vangl2 and Tmem14a may have opposing roles in regulating Golgi structure, and the two together may need to be co-expressed in order for the structure of the trans-Golgi network to be normal.

2.13 Discussion

I have shown here using fluorescence tagging and morpholino approaches that Tmem14a is a potential regulator of PCP signaling. Tmem14a is conserved in vertebrate species but not in Drosophila; thus, the findings of this study are relevant to vertebrate-specific PCP processes. \textit{tmem14a} mRNA is expressed throughout zebrafish development. Tmem14a-GFP localizes to the trans-Golgi network as well as to the base of cilia. \textit{tmem14a} morphants display curved body axes, a phenotype that is associated with defects in apical/basal polarity and ciliogenesis. Furthermore, morphants also display a convergent extension defect and an ectopic accumulation of cells in the neural tube, phenotypes that are associated with planar cell polarity. An analysis of cilia in the neural tube shows severely disorganized cilia, similar to MZvangl2 mutants. Based on its localization in the trans-Golgi network, I hypothesized that Tmem14a may have a role in
Figure 24. Overexpression of GFP-Vangl2 in HEK293T cells causes Golgi scattering, which is rescued by co-expression of Tmem14a-myc. Co-expression of Tmem14a-myc restores normal Golgi condensation around the nucleus. The top panels show that GFP-Vangl2 is membrane localized, the middle panels show that GFP-Vangl2 is localized to both the membrane and Golgi. The bottom panels show that GFP-Vangl2 is completely localized to the Golgi.
Figure 25. Co-expression of GFP-Vangl2 and Tmem14a-myc in HEK293T cells causes retention of GFP-Vangl2 in the trans-Golgi network. Cells overexpressing only GFP-Vangl2 show a greater percentage in the membrane localization of GFP-Vangl2. When both GFP-Vangl2 and Tmem14a-myc were overexpressed, a greater percentage of cells showed localization of GFP-Vangl2 to both the membrane and the trans-Golgi network.
trafficking Vangl2 to the membrane, and have shown that tmem14a morphants indeed have defects in GFP-Vangl2 membrane localization. Using HEK293T cells, I have shown that overexpression of GFP-Vangl2 results in the scattering of the trans-Golgi network. This scattering is rescued by co-expression of Tmem14a-myc, possibly by interacting with and trapping GFP-Vangl2 in the trans-Golgi network. Furthermore, overexpression of Tmem14a-myc or Tmem14a-GFP in HEK293T cells resulted in the polarization of the trans-Golgi network towards one side of the cell. This suggests that expression of Tmem14a alone may be sufficient to polarize HEK293T cells, which are normally non-polarized.

Together, these results suggest that Tmem14a has a potential role in regulating PCP but it is also possible that Tmem14a has a role in regulating apical/basal polarity. Additional experiments are needed to define the role of Tmem14a in regulating PCP, apical/basal polarity, and ciliogenesis. These experiments will be discussed in the next chapter.

The data presented in this study suggest several possible models for the function of Tmem14a. Tmem14a could be involved in the trafficking of Vangl2 from the ER to the Golgi, or through the trans-Golgi network. One possible role for Tmem14a is that it could act as a chaperone for Vangl2 as it is being trafficked. By interacting with and guiding Vangl2 through the ER or Golgi, Tmem14a could prevent Vangl2 from being targeted to non-specific sites within the cell. Another possible function for Tmem14a is in the regulation of Golgi morphology. Tmem14a could be involved in maintaining the elongated structure of the trans-Golgi network such that it is tethered to a specific subcellular structure beneath the apical membrane, such as the basal body of cilia. In this way, Tmem14a could regulate the positioning of the basal body beneath the apical membrane and hence the tilt of the cilia. Furthermore, since it is associated with the base of cilia, Tmem14a could regulate the trafficking of proteins into cilia, similar to other tetraspanins such as Tmem231 and Tmem175. I demonstrated that knockdown of Tmem14a mRNA activity resulted in greater cytoplasmic localization of GFP-Vangl2. However, it is possible that GFP-Vangl2 was endocytosed more rapidly, rather than there being a defect in the trafficking of GFP-Vangl2. If this is the case, then a possible role for Tmem14a could be in maintaining GFP-Vangl2 stably at the membrane and preventing it from being endocystosed.

The results of this study show that GFP-tagged Tmem14a is localized to the trans-Golgi network and not to the mitochondria. However, one previously published paper reported that the
subcellular localization of Tmem14a is in the mitochondria. One possible reason for this discrepancy is that the GFP tag is interfering with the proper localization of Tmem14a. For example, it could affect the folding of the protein or the trafficking of Tmem14a. Hence, it would be better to generate an antibody that could detect the localization of endogenous Tmem14a. However, this would be difficult since there are very few amino acids on the cytosolic surface that could be used as an antigen to generate an antibody. An alternative would be to determine the localization of myc-tagged Tmem14a using immunofluorescence since the myc tag is smaller than the GFP tag and would have less chance of interfering with the folding or trafficking of the protein. It would also be useful to rescue tmem14a morphants using Tmem14a-myc or Tmem14a-GFP mRNA. This would give further evidence that the tagged protein is functional and targeted to its proper subcellular localization.

Another reason for the discrepancy between this study and the study done by Woo et al. (2011) is that two different systems were used to draw conclusions about the localization of Tmem14a. For example, Woo et al. (2011) characterized the localization of Tmem14a in vitro and used HeLa cells whereas zebrafish embryos were used in this in vivo study. In addition, the method of labeling the mitochondria was different. Woo et al. (2011) used the mitochondrion-specific dye MitoTracker to stain mitochondria in cell culture whereas this study used a GFP construct that is targeted to the mitochondria through the mitochondrial localization signal sequence from human cytochrome c oxidase. As a result, this may cause the morphology of the mitochondria to appear different: in the study done by Woo et al. (2011), the mitochondria appears diffuse and cytoplasmic whereas this study shows that the mitochondria are discrete, rounded structures in the cytoplasm. The reliability of the MitoTracker in labeling the mitochondria is also questionable since the staining may be background staining. The picture quality of the data was also poor: the MitoTracker also appeared to co-label with the ER, and a Golgi stain was not used to determine whether Tmem14a-GFP co-localized with the Golgi.

Because of their different localizations, different conclusions were drawn about the function of Tmem14a. For instance, Woo et al. (2011) concluded that Tmem14a is localized to the mitochondria and suppresses apoptosis by inhibiting Bax, which functions in proapoptotic events. In this study, I concluded that Tmem14a is localized to the Golgi and is involved in Vangl2 trafficking. Despite the fact that different conclusions were drawn with regards to the main localization of Tmem14a, both studies suggested that Tmem14a may also be localized to
the ER. This is significant because both the mitochondria and the Golgi can associate with the ER. For example, it has been reported that the mitochondria can be physically tethered to the ER by protein complexes\textsuperscript{70}. These protein complexes, termed the ER-mitochondria encounter structure (ERMES), are essential for mitochondrial inheritance and movement, replication of mitochondrial DNA, and trafficking of proteins into the mitochondria\textsuperscript{70}. Similarly, the ER is involved in Golgi biogenesis, a process that involves constant trafficking of vesicles in the anterograde and retrograde direction between the ER and the Golgi\textsuperscript{71}. Thus, Tmem14a protein that is localized to the ER can be closely associated with both the mitochondria and the Golgi.

Overexpression of Tmem14a-myc in HEK293T cell culture and knockdown of Tmem14a activity in zebrafish both result in the same Vangl2 trafficking defect. This may be because levels of Tmem14a expression plays a role in determining where Vangl2 is destined. For example, overexpressing Tmem14a-myc at high levels in HEK293T cells may result in the interaction and trapping of GFP-Vangl2 in the trans-Golgi network, whereas knockdown of Tmem14a activity decreases the amount of Tmem14a protein that can guide Vangl2 through the Golgi, which results in GFP-Vangl2 being retained in the ER or Golgi through other Tmem14a-independent mechanisms. Degradation of the trapped GFP-Vangl2 may have resulted in the cytoplasmic GFP seen in Tmem14a morphant embryos.

In summary, the MYTH screen has identified a novel vertebrate-specific regulator of PCP with possible functions in the localization and trafficking of core PCP components in addition to functions in cilia regulation. A full analysis of genetic \textit{tmem14a} mutant phenotypes should provide tremendous insight into Tmem14a function in cell polarity and vertebrate embryonic morphogenesis.

2.14 Generation of \textit{tmem14a} mutant zebrafish

There are several disadvantages of using morpholinos to study mutant phenotypes. For example, morpholinos can cause nonspecific activation of the p53 pathway, which can be toxic to embryos\textsuperscript{72}. Furthermore, the morpholino may not completely eliminate gene products because of the efficiency at which the morpholino blocks translation of the targeted RNA. Hence, the mutant phenotype of the morphant may be only partially penetrant. Splice morpholinos are also ineffective at knocking down maternally deposited mRNA since these are already fully spliced.
Figure 27. Zinc finger nucleases bind to target DNA sequences to generate mutations through DNA repair. (A) ZFNs function as dimers. Each monomer binds to a 9 bp region flanking the target site. The binding of ZFN as dimers activates the nuclease domain, which cleaves the DNA in the spacer region. Non-homologous end joining results in the addition or elimination of nucleotides in the spacer region, generating indel mutations (from Foley, J.E. et al. (2009)). (B) Target DNA site for the \textit{tmem14a} locus. One ZFN binds to the left site located in intron 2 while the second ZFN binds to the right site located in exon 3. The spacer site is located in the junction between intron 2 and exon 3.
When using morpholino, there is a range of phenotypes that is observed because mRNA is constantly being transcribed and translated, which lowers the effectiveness at which the morpholino can target existing RNA. Because of the range of phenotypes observed, it is uncertain which phenotype represents the true loss of function phenotype. Hence, the maternal-zygotic phenotype can only be analyzed using heritable mutations.

To generate heritable mutations of zebrafish \textit{tmem14a}, I used specifically engineered zinc finger nucleases (ZFNs). ZFN technology aims at constructing zebrafish mutants using ZFNs to introduce mutations at the target locus\textsuperscript{73,74}. ZFNs bind to the target locus as dimers (figure 27). Each monomer consists of the endonuclease portion of the \textit{FokI} enzyme fused to an engineered zinc finger array that binds to the target DNA sequence. The zinc finger array consists of three or four zinc fingers, and each zinc finger binds to a 3 bp site. Thus, one monomer binds to a 9 bp or 12 bp site at the left side of the target site and the second monomer binds to a 9 bp or 12 bp site at the right side of the target site. Cleaving of the DNA by the ZFNs activates the DNA repair machinery, which can potentially add or excise extra nucleotides during nonhomologous end joining repair to create a mutation in the target site. Oligomerized Pool ENgineering (OPEN) has been used to generate zinc finger arrays that are designed to target a specific DNA sequence\textsuperscript{73,74}. The OPEN system is a pool of mutagenized zinc finger nucleases with random specificities. To generate the ZFNs, the OPEN pool is used in PCR reactions that will stitch together random ZFNs into a tandem array. The PCR reaction is then subcloned into a bacterial one-hybrid system that will activate a reporter gene if the ZFN binds to the target DNA sequence. The stringency of the selection medium can be changed to select for ZFNs that bind to the target DNA sequence with lower or higher specificities.

To generate the zinc-finger nuclease mutant, RNA encoding zinc finger nucleases were designed by others in the lab and then injected into wild-type embryos at the one cell stage. The ZFNs were designed to bind to the \textit{tmem14a} locus on both sides of the intron 2/exon 3 boundary (figure 27). To detect whether the \textit{tmem14a} locus was mutated, the \textit{tmem14a} locus was PCR amplified from genomic DNA, and the PCR products were digested using a restriction enzyme that recognizes the target site within the ZFN target region (figure 28). Indel mutations are expected to remove the target site. Since not all cells in the embryo are expected to efficiently target the \textit{tmem14a} locus, incomplete digest of the PCR product will indicate that the ZFNs has targeted the locus in some cells of the embryo. I have raised several hundred ZFN-injected
embryos to adulthood and have tail-clipped and screened 175 fish for a mutation at the \textit{tmem14a} locus. I have identified 27 potential founders out of the 175 fish. The PCR screen showed that these potential founders may carry a somatic \textit{tmem14a} mutation in the tail (figure 28). To identify founders that carry a \textit{tmem14a} mutation in the germline, I am currently outcrossing the potential founders to wild-type fish and screening the embryos for heterozygous mutants. Of the 27 potential founders, I have outcrossed nine potential founders and have not yet identified any heterozygous mutant progeny from the outcrosses (figure 29; table 1).

2.15 Methods and Materials

**Co-immunoprecipitation and Western Blotting**

Transfected HEK293T cells were lysed with HEPES lysis buffer (20 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EGTA pH 8.0, 10 mM NaF, 10 mM sodium pyrophosphate, 25 mM β-glycerol phosphate, 1 mM sodium orthovanadate) with protease inhibitors (SIGMA) and incubated with anti-myc antibodies (Santa Cruz) at 4°C overnight followed by incubation with Dynabeads (Invitrogen) at 4°C overnight. Dynabeads were washed three times in lysis buffer and immunoprecipitates were eluted with sample buffer, then analyzed using standard western blot procedures.

**Reverse-transcriptase PCR**

Fifty staged embryos were dechorionated and total RNA was extracted using Trizol reagent (Invitrogen). First-strand cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen) and Oligo (dT)\textsubscript{12-18} primer (Invitrogen). To analyze the expression of Tmem14a mRNA, the mRNA was amplified using the following primers: Forward: 5’-CTTCAACTTTTGTAACGCAGCGCA-3’ and Reverse: 5’-ACAAGATGGCAGTGGAAGAAAGCA-3’. PCR amplification was performed using Taq polymerase (New England Biolabs) and the following protocol: 95°C for 5 min, 35 cycles of 95°C for 40 s, 58°C for 40 s, 72°C for 40 s, and 72°C for 10 min.
Figure 28. Potential founders are identified using tail clipping and PCR. ZFN-injected fish were raised to adulthood and their tails were clipped. Genomic DNA from tail clips were used in PCR reactions that amplified the ZFN target site within the tmem14a target locus. An incomplete digest (i.e. the presence of an undigested band; arrows) indicates that there is a potential mutation in the target site. (Undigested band = 430 bp; digested bands = 104 bp + 326 bp). Individual potential founders are numbered in each lane.
Figure 29. Complete enzymatic digest of PCR reactions show that the potential founder does not carry a ZFN mutation in the germline. To identify ZFN mutants among the potential founders, founders were outcrossed to wild-type fish and embryos were lysed to isolate genomic DNA. The DNA were used in PCR reactions that amplified the target site within the *tmem14a* locus. A complete digest indicates that there are no heterozygous mutant embryos, and that the potential founder does not carry a mutation in the germline. (Digested bands = 104 bp + 326 bp). Each lane represents an enzymatic digest from one embryo. The above gels show enzymatic digests from a total of 96 embryos obtained from one outcross.
### Table 1. Outcross of potential founders to wild-type fish and the number of progeny screened for each outcross.

<table>
<thead>
<tr>
<th>Potential founder</th>
<th>Date of birth</th>
<th>Outcrossed to</th>
<th>Number of progeny screened</th>
<th>% Heterozygous mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>#5 male</td>
<td>09/05/2010</td>
<td>WT female</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
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<td>09/05/2010</td>
<td>WT female</td>
<td>168</td>
<td>0</td>
</tr>
<tr>
<td>#66 male</td>
<td>09/05/2010</td>
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<td>94</td>
<td>0</td>
</tr>
<tr>
<td>#84 male</td>
<td>09/20/2010</td>
<td>WT female</td>
<td>96</td>
<td>0</td>
</tr>
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<td>09/20/2010</td>
<td>WT female</td>
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<tr>
<td>#125 male</td>
<td>10/15/2010</td>
<td>WT female</td>
<td>112</td>
<td>0</td>
</tr>
</tbody>
</table>
Plasmids
GFP-Vangl2 (Madeline Hayes and Jovana Drinjakovic, University of Toronto), Tmem14a-GFP (Brian Ciruna, University of Toronto), Tmem14a-myc, GFP-Tmem14a, untagged Tmem14a, DsRed-GalT (Clontech) plasmids were constructed using Gateway cloning technology (Invitrogen)^75,76. Middle entry vectors encoding Vangl2 (Madeline Hayes and Jovana Drinjakovic), Tmem14a (Brian Ciruna), or GalT (Clontech) were used in recombination reactions with either 5’ entry vectors encoding GFP (Nathan Lawson) or DsRed (Nathan Lawson) or 3’ entry vectors encoding GFP (Nathan Lawson), myc (Madeline Hayes) or polyA tail (Chi-Bin Chien).

Morpholinos and mRNA microinjections
Live embryos were injected with morpholino or mRNA at the one-cell stage. The translation blocking morpholino (Tmem14a ATG-MO; 5’-CCAGCCAGTCCACAGCCATCACAAC-3’) and splice blocking morpholino targeting the exon 2/intron 2 boundary (Tmem14a exon 2; 5’-GCAGAACAAAAATCCCTCCATACTTT-3’) were obtained from Gene Tools, LLC. Embryos were injected with 2 ng, 3 ng, 3.5 ng, or 4 ng of ATG morpholino, and 2 ng, 4 ng, or 6 ng of splice morpholino. mRNA was synthesized in vitro using mMESSAGE mMACHINE kit (Ambion) and injected into embryos at the one-cell stage. Embryos were injected with 100 pg of GFP-Vangl2, 20 pg of Tmem14a-cherry, 20 pg of Tmem14a-GFP, 20 pg of GFP-Tmem14a, 20 pg of Tmem14a-myc, 15 pg of memb-RFP.

Confocal microscopy
Live embryos were mounted in 0.8% low melt agarose and confocal images were taken using 63X objective (Zeiss 710). Z stacks were taken at 1 μm intervals.

Whole-mount RNA in situ hybridization
Whole-mount RNA in situ hybridization was performed using standard procedures. Krox20 and myoD riboprobes were synthesized using DIG RNA Labeling Kit (Roche), digoxigenin-11-UTP, and T3 polymerase. Stained embryos were mounted in benzylbenzoate/benzylalcohol and imaged using Axio Imager (Zeiss).
Co-localization analysis
ImageJ software was used to quantify the co-localization between memb-RFP and GFP-Vangl2. The channels were split into red and green for each image and the JACoP plugin was used to calculate Mander’s coefficient after setting a threshold value for each channel. Three z stacks were chosen for each embryo and four thin rectangles were outlined for each z stack. The image was cropped and the channel was split again. The threshold value was set to eliminate background fluorescence and Mander’s coefficients were determined. The average Mander’s coefficient and standard deviation for wild-type versus morpholino-injected embryos was calculated using Microsoft Excel.

HEK293T cell culture and cell transfections
HEK293T cells were maintained in 5% CO2 in Dulbecco’s medium supplemented with 5% penicillin/streptomycin. Cells were passaged once every three days using trypsin. Lipofectamine transfection reagent (Invitrogen) was used according to the manufacturer’s protocol. 2.5 ug of each plasmid was transfected at 50% confluence and the medium was changed the next day. After allowing cells to grow for another day, cells were used for co-immunoprecipitation or confocal microscopy.

Zinc finger nuclease mutagenesis
Plasmids encoding two ZFNs (Mizue Naito, Hospital for Sick Kids Research Institute, Toronto) were injected into embryos at the one-cell stage at a concentration of 70 pg each. Embryos were raised to 3 months and tails were clipped. Tail clippings were heated to 98°C in 50 mM NaOH in a PCR machine and then neutralized using 1 M Tris pH 8.0. Genomic DNA was amplified using the following primers: Forward: 5’-TGGTTCAAAGACTTGATTGTG-3’ and Reverse: 5’-TGGTGATCTTCCACAACATGGA-3’. PCR amplification was performed using Taq polymerase (New England Biolabs) and the following protocol: 95°C for 5 min, 35 cycles of 95°C for 30 s, 58°C for 30 s, 72°C for 30 s, and 72°C for 10 min.
Chapter 3
Future Directions

3 Future Directions

3.1 Further analysis of Tmem14a mutant phenotype

Tmem14a is a potential regulator of planar cell polarity and Vangl2 trafficking. *tmem14a* morphants exhibit convergent extension defects and an ectopic accumulation of cells in the neural tube, supporting that Tmem14a is a potential regulator of PCP signaling. However, Tmem14a could also potentially regulate apical/basal polarity and ciliogenesis since the curved body axes exhibited by *tmem14a* morphants is a phenotype that can be caused by defects both in apical/basal polarity and ciliogenesis. Several experiments can be done to further understand whether *tmem14a* morphants can be classified as a planar cell polarity, apical/basal polarity or ciliogenesis mutant. In addition, it would be necessary to generate a maternal-zygotic *tmem14a* mutant to analyze complete loss of function phenotypes.

3.2 Examine whether knockdown of Tmem14a activity results in trapping of GFP-Vangl2 in the ER or the Golgi

The presence of perinuclear GFP-Vangl2 puncta could be detected in the neuroepithelial cells of *tmem14a* morphants (figure 20). These puncta may be budding ER vesicles that fail to be transported to the Golgi, suggesting that knockdown of Tmem14a activity does not affect the sorting and packaging of GFP-Vangl2 into ER vesicles, but disrupts the transport of the ER vesicles to the Golgi. To test this hypothesis, the ER can be labeled and the GFP-Vangl2 puncta can be examined to determine whether they coincide with the ER. These results can be confirmed by quantifying the overlapping GFP and mCherry signals using imaging analysis software. The number of GFP-Vangl2 puncta found in the ER can be compared to those found in wild-type embryos. Knockdown of Tmem14a activity can also result in trapping of GFP-Vangl2
in the Golgi. This result can be further investigated by labeling the Golgi with DsRed-GalT and examining whether the DsRed-GalT signal overlaps with the GFP signal, using imaging analysis software. The results of these experiments will determine whether knockdown of Tmem14a activity causes trapping of GFP-Vangl2 in the ER or Golgi.

3.3 Defects in planar cell polarity

To further examine whether Tmem14a regulates all known functions of PCP signaling, it will be necessary to examine defects in cell intercalation and migration since PCP signaling is needed for the migration and re-intercalation of neuroepithelial cells into the neuroepithelium after midline crossing. To examine whether knockdown of Tmem14a activity affects midline crossing and cell intercalation, embryos can be injected with photoconvertible Kaede mRNA and one side of the neural tube can be labeled by photoconverting with UV light. Time lapse microscopy can be used to examine neuroepithelial cells as they divide, and dividing cells can be examined for whether they can cross the midline after cell division. If cells do not cross the midline and reintercalate into the neuroepithelium after cell division, this indicates that the Tmem14a morphant phenotype is caused by a defect in PCP signaling.

Another indication of a PCP defect is the localization of Prickle, a core PCP component. Prickle is asymmetrically localized to the anterior membrane of neuroprogenitors during the neural keel stage. MZvangl2 embryos show a loss in the asymmetric localization of GFP-Prickle. Using cell transplantation, the localization of GFP-Prickle can be examined in Tmem14a ATG-MO donor cells. If the asymmetric localization of GFP-Prickle is disrupted, this suggests that Tmem14a plays a role in establishing PCP in neuroepithelial cells.

Vangl2 is required for asymmetric basal body localization, which results in the positioning of cilia on the posterior side of floorplate cells. To further investigate whether PCP signaling is disrupted, the localization of Centrin-GFP can be examined, which labels the basal body, in Tmem14a ATG-MO donor floorplate cells. The number of basal bodies that are docked centrally, as seen in MZvangl2 mutants, versus the number of basal bodies that are docked on the anterior side of floorplate cells can be quantified. If a significant number of basal bodies are docked centrally, this suggests that PCP signaling is disrupted by knockdown of Tmem14a activity.
Finally, PCP signaling also regulates the migration of facial branchiomotor (FBM) neurons in the zebrafish hindbrain\cite{77}. FBM neurons are first born in the fourth rhombomere (r4) and undergo directed cell migration to r6 and r7; cell-cell communication between the migrating neuron and the neuroepithelial environment regulates directed cell motility\cite{77}. In the zebrafish, zygotic vangl2 mutants as well as other core PCP mutants such as Prickle and Frizzled 3 exhibit defects in FBM neuronal migration\cite{60,78,79}. To determine whether Tmem14a regulates FBM neuronal migration, Tmem14a ATG-morpholino can be injected into transgenic embryos expressing GFP in branchiomotor neurons and neurons can be examined for migration from r4 to r6 and r7 using confocal microscopy. If the facial branchiomotor neurons show a defect in cell migration, then Tmem14a may play a role in regulating PCP processes needed for neuronal migration.

3.4 Defects in apical/basal polarity

The ectopic accumulation of cells in the neural tube can be caused either by PCP-regulated defects in cell migration and intercalation\cite{5}, or by apical/basal polarity defects in oriented cell division\cite{80}. *tmem14a* morphants show a curved body axis at 48 hpf. Mutations in genes that regulate apical/basal polarity, such as heart and soul/apPKC\cite{81}, the crumbs complex (nagie oko\cite{66} and oko meduzy\cite{82}) Bazooka/Pard3\cite{83}, and Pard6\cite{84} also cause curved body axes. Given that Vangl2 and Scribble interact\cite{43}, this suggests that there is a possible connection between the regulation of apical/basal polarity and planar cell polarity. Therefore, it will be interesting to investigate whether Tmem14a plays a role in regulating apical/basal polarity.

As neuroepithelial cells acquire apical/basal polarity during the neural keel stages (10-12 somites), Pard3-GFP becomes enriched at the apical membrane of cells at the midline\cite{80}. To examine whether apical/basal polarity is disrupted by knockdown of Tmem14a activity, Tmem14a ATG-MO donor cells can be labeled with Pard3-GFP to examine the localization of Pard3-GFP in wild-type hosts at the neural keel and neural rod stages. A disruption in Pard3-GFP localization suggests that knockdown of Tmem14a activity causes defects in apical/basal polarity. If there is a defect in Pard3-GFP localization, knockdown of Tmem14a activity results in apical/basal polarity defects can be further confirmed by using immunofluorescence staining.
of the apical markers aPKC and ZO-1. Localization of these markers can be examined at neural rod stages (15 somite stage), when aPKC and ZO-1 are expressed at the apical midline. During the neural keel stages, division of neuroprogenitors across the midline depends on the proper orientation of the mitotic spindle in the apical/basal axis. To examine whether oriented cell division is disrupted, embryos can be injected with Histone2B-GFP mRNA to label chromosomes, and confocal time-lapse microscopy can be used to monitor cells in mitosis. The number of cells that divide in the correct orientation in tmem14a morphants can be quantified and compared to wild-type controls. A significant deviation from the number of cells that divide in the correct orientation may indicate Tmem14a plays a role in regulating apical/basal polarity.

3.5 Defects in ciliogenesis

My preliminary data suggests that knockdown of Tmem14a results in disorganized cilia, similar to MZvangl2 mutants. In wild-type embryos, normal fluid flow is generated by the counterclockwise motion of cilia in Kupffer’s vesicle (KV). To investigate whether ciliary motion is disrupted by knockdown of Tmem14a activity, fluorescent beads can be injected into the KV of tmem14a morphants and their motion can be monitored in 8-10 somite stage embryos using time-lapse microscopy. If the fluid flow is disrupted in the KV, this may be due to disruptions in the posterior tilt of the cilia. To investigate this, cilia can be labeled in Tmem14a morphants using Arl13b-GFP and the KV can be imaged in 6-8 somite stage embryos using confocal microscopy. The number, length, and range of motion of cilia in the KV can be quantified using image analysis software. To further confirm the cilia phenotype seen in the KV, Tmem14a ATG-MO donor cells can be transplanted into the floorplate of the neural tube in wild-type hosts at 24 hpf and the tilt of cilia can be quantified. To show that the cilia defects are not artifacts caused by injecting Arl13b-GFP, Arl13b-GFP transgenic embryos can also be used.

Proper left/right patterning depends on the fluid flow generated by the ciliary motion in Kupffer’s vesicle. Lefty expression is restricted to the left lateral plate mesoderm of the embryo during gastrulation and this expression pattern can indicate that proper left/right patterning is established. To confirm that ciliary function is compromised in tmem14a morphants, Lefty mRNA expression can be examined in 20-22 somite stage embryos using in situ hybridization.
If Tmem14a plays a role in ciliogenesis, it is possible that cilia will fail to form at all in *tmem14a* morphants. To examine this, confocal microscopy will be used first to analyze cilia formation. If short, stumpy cilia are observed, then electron microscopy will be used next to determine whether the basal bodies are docked, but cilia fail to form. The pronephric duct will also be examined to determine whether Tmem14a functions in the formation of different types of primary cilia.

### 3.6 Can Tmem14a interact with the Looptail mutated variants of Vangl2?

The two classical Looptail mutations (S464N and D255E) in Vangl2 were identified using forward genetic screens\(^85,86\). Both mutations are semi-dominant and map to the C-terminus of Vangl2\(^85,86\). In the mouse, a heterozygous mutation causes looped tails whereas a homozygous mutation causes craniorachischisis\(^85,86\). Furthermore, these mutations cause Vangl2 to be trapped in the ER\(^48\). In addition, both Vangl2\(^D255E\) and Vangl2\(^S464N\) fail to be packaged into COPII vesicles in in vitro budding assays, suggesting that Vangl2 proteins that harbor a Looptail mutation cannot interact with Sec24b\(^49\). Therefore, it is possible that Vangl2\(^D255E\) and Vangl2\(^S464N\) are trapped in the ER because the Looptail mutations abolish the interaction between Vangl2 and Tmem14a. To test this, Vangl2\(^S464N\) and Vangl2\(^D255E\) can be cloned in frame with a GFP tag and their localization in HEK293T cells can be examined to confirm that they are trapped in the ER. Co-immunoprecipitation can then be performed to detect whether Vangl2\(^S464N\) and Vangl2\(^D255E\) can interact with Tmem14a-myc. If Vangl2\(^S464N\) and Vangl2\(^D255E\) do not co-immunoprecipitate with Tmem14a-myc, this suggests that Vangl2\(^S464N\) and Vangl2\(^D255E\) are trapped in the ER because they cannot interact with Tmem14a. This experiment will further confirm the role of Tmem14a in trafficking Vangl2 from the ER to the Golgi.

### 3.7 Interaction of Tmem14a with Sec24b

In mice, it has been shown that Sec24b sorts Vangl2 into COPII vesicles for ER to Golgi trafficking\(^49\). Tmem14a may interact with Sec24b in the ER to promote the sorting of Vangl2; to
test this interaction, the zebrafish Sec24b can be cloned in frame with a GFP tag and co-
immunoprecipitation can be performed by co-expressing Sec24b-GFP and Tmem14a-Myc in
HEK293T cells. If Tmem14a interacts with Sec24b, this suggests a possible mechanism for the
trafficking of Vangl2 by Tmem14a. It will also be useful to perform a MYTH screen to identify
potential interactors of Tmem14a, which can provide clues about the pathways that Tmem14a
participates in.

3.8 Is Vangl2 internalized more rapidly after knockdown of Tmem14a activity?

Another explanation for the reduced membrane localization and presence of GFP-Vangl2
in the cytoplasm is that knockdown of Tmem14a activity results in the rapid endocytosis of GFP-
Vangl2 at the membrane, rather than a role for Tmem14a in trafficking GFP-Vangl2 to the
membrane. This possibility can be tested by inhibiting endocytosis using chlorpromazine\(^7\) and
determining whether this rescues the phenotype, i.e. restores GFP-Vangl2 to the membrane.
First, embryos will be co-injected with GFP-Vangl2, membrane-RFP, and Tmem14a ATG-
morpholino. The embryos will be grown in media containing chlorpromazine from the one-cell
stage to the 16 somite stage. If the media is too toxic, then the embryos will be grown in the
chlorpromazine at a later stage in development. Then, the co-localization of GFP-Vangl2 with
membrane-RFP will be analyzed using confocal microscopy. If GFP-Vangl2 significantly co-
localizes with membrane-RFP and the co-localization co-efficient is similar to wild-type, then it
can be concluded that inhibition of endocytosis causes GFP-Vangl2 to remain at the cell
membrane. This experiment will distinguish whether Tmem14a is required for GFP-Vangl2 to be
stably expressed at the membrane, rather than for trafficking GFP-Vangl2 to the membrane.

3.9 A role for PCP signaling in regulating Golgi structure and polarity

The in vitro data shown in this study demonstrated that the Golgi appeared scattered
when GFP-Vangl2 was overexpressed (figure 23), and that Tmem14a-GFP overexpression
polarized the Golgi towards one side of the cell (figure 22). This suggests that GFP-Vangl2 and
Tmem14a-GFP have opposing functions with regards to Golgi structure and polarity. To further
explore the roles of Vangl2 and Tmem14a in regulating Golgi structure and polarity, the Golgi can be examined in maternal-zygotic vangl2 mutants. First, MZ vangl2 mutants embryos can be co-injected Arl13b-GFP and DsRed-GalT. Mutant cells can be transplanted into wild-type hosts and confocal microscopy can be used to determine whether the trans-Golgi network is polarized towards the base of the cilia. A similar experiment can be done using tmem14a morphants in place of MZ vangl2 mutants. If the Golgi does not extend towards the base of the cilia, this indicates that PCP signaling and Tmem14a play a role in structuring the Golgi such that it contacts the base of cilia.
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