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Development of Planar Cell Polarity in the Mammalian Vestibular Sensory Organs

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Abstract

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By Jack Nixon Etheredge

Epithelial planar cell polarity (PCP) refers to the coordinated orientation of neighboring cells, resulting in a polarity axis parallel to the plane of the epithelium. The vertebrate PCP pathway consists of a set of membrane-associated proteins, and primary cilia and their associated basal bodies. The underlying mechanism in vertebrate PCP signaling, however, has yet to be revealed. Each sensory hair cell of the inner ear contains a polarized hair bundle comprising an asymmetrically positioned primary cilium, the kinocilium, and rows of microvilli-derived stereocilia of graded height. Hair cells within each inner ear organ are oriented coordinately. In particular, the hair cells in the saccule and utricle show reverse polarity along a line of polarity reversal, providing a unique opportunity to dissect PCP signaling. To explore the mammalian maculae as PCP models, we established the timeline for cell proliferation, differentiation, and hair bundle formation in the mouse maculae. We further analyzed the subcellular localization of membrane associated PCP proteins, Vang-like 2 (Vangl2) and Frizzled 3 (Fz3). We found asymmetric and polarized membrane distribution of Vangl2 and Fz3. Moreover, asymmetric distribution of both Fz3 and Vangl2 precedes formation of stereocilia. Similar to published results of another PCP protein Pk2, confocal microscopy of embryonic day (E)18 mouse embryos shows that Vangl2, or Vangl2-GFP, and Fz3 appear to colocalize on one side of the boundary formed by a sensory hair cell and the adjacent non-sensory supporting cell in both maculae, independent of the orientation of the hair cells. Adenoviral transfection of eGFP-Vangl2 fusion protein in cultured utricles and saccules showed that Vangl2 can be enriched in both the hair cell and the supporting cell to the same boundary. These observations suggest that PCP proteins are distributed asymmetrically in both sensory hair cells and their neighboring supporting cells, and that the polarity of PCP proteins alone is not sufficient to determine the orientation of hair cells.

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List of Abbreviations

AC: Anterior cristae

Dsh: Dishevelled

Dsh3: Dishevelled 3

E: Embryonic day

Fzd: Frizzled

Fzd3: Frizzled 3

Fzd6: Frizzled 6

GFP: Green fluorescent protein

L: Lateral (side of the organ of Corti or vestibular maculae)

LC: Lateral cristae

M: Medial (side of the organ of Corti or vestibular maculae)

PC: Posterior cristae

PCP: Planar cell polarity

Pk: Prickle

Pk2: Prickle 2

SA: Saccule

UT: Utricle

Vang: Van Gogh

Vangl2: Van Gogh-like 2

WT: Wild type

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Chapter 1 General Introduction

Planar Cell Polarity

Planar cell polarity (PCP) in epithelial tissues refers to the coordinated orientation of the neighboring cells resulting in a polarity axis parallel to the plane of the epithelial sheet (Vinson and Adler, 1987). The sensory organs of the mammalian inner ear each exert distinct forms of PCP. Each sensory hair cell of the inner ear contains a polarized hair bundle comprising an asymmetrically positioned primary cilium known as the kinocilium and rows of microvilli-derived stereocilia of graded height. All of the hair cells in each sensory organ of the inner ear are oriented coordinately with respect to the asymmetrically positioned kinocilium and stereocilia. The distinct PCP in the organ of Corti in the cochlea is necessary for audition while PCP in the five sensory organs of the vestibule is, presumably, necessary for balance.

Past studies have revealed the morphological events and critical components of the vertebrate PCP pathway in establishing PCP in the cochlea. In particular, primary cilia and/or their associated basal bodies have been identified as novel components of the vertebrate PCP pathway essential for cochlear development (Jones and Chen 2008). However, the morphological events leading to the formation of PCP in the vestibular sensory organs are not characterized and the underlying molecular mechanism has yet to be revealed.

Intrinsic Cellular Polarity

Intrinsic cellular polarity is necessary for establishing coordinated planar cell polarity, whether the planar cell polarity is expressed in mesenchymal cells as in convergent extension processes or in epithelial cells as in the *Drosophila* wing bristles or sensory hair cells of the mammalian inner ear. During development, convergent extension refers to embryonic tissue simultaneously narrowing along one axis and elongating along a perpendicular axis. This is achieved by the movement of cells and does not require cellular growth. One example of convergent extension is neural tube formation. Intrinsic cellular polarity refers to the asymmetric localization of some cellular chemical or structure, usually a protein or organelle. Thus, a cell can have normal intrinsic polarity and disrupted planar cell polarity if there is normal asymmetric localization of structures within the cell relative to other structures within the same cell, but the asymmetric localization is not coordinated with the surrounding cells in the tissue (Figure 1.1).

Invertebrate PCP Signaling

PCP signaling requires directional cues for the cell field, cellular factors to interpret spatial information and establish the primary PCP axis for coordination among neighboring cells, and cell-specific downstream effectors that create the polar structure of individual cells (Vinson and Adler 1987; Vinson, Conover et al. 1989). A set of evolutionarily conserved genes, known as core PCP genes, acts

to establish an axis for PCP to direct cellular polarity. Core PCP genes comprise a set of trans-membrane proteins and their immediate downstream effectors. The known core PCP proteins include the transmembrane proteins Van Gogh/Strabismus (Vang) (Taylor, Abramova et al. 1998; Wolff and Rubin 1998), Flamingo (Usui, Shima et al. 1999), Frizzled (Fz) (Vinson and Adler 1987; Vinson, Conover et al. 1989), and the cytoplasmic protein Dishevelled (Dsh/Dvl) (Klingensmith, Nusse et al. 1994; Theisen, Purcell et al. 1994), Diego (Dgo) (Feiguin, Hannus et al. 2001), and Prickle (Pk) (Gubb, Green et al. 1999).

PCP is readily observed in *Drosophila* as the coordinated orientation of hairs that grow from the epithelial cells and point either toward the posterior on the body or distally on the wing. A role for core PCP proteins in the regulation of PCP was first identified in studies of mutants that caused a disruption in the polarization of the hair bristles in the *Drosophila* wing (Vinson and Adler 1987). PCP proteins are asymmetrically sorted in a distinct and robust pattern reflecting the polarity of the cell before hair growth. In the epithelial cells of the wing, Fz and Dsh accumulate along the distal edge of the cells and Vang and Pk accumulate on the proximal side of cells (Adler 2002; Tree, Shulman et al. 2002; Klein and Mlodzik 2005). This interaction between membrane PCP proteins at the opposing boundary formed by the neighboring cells is thought to underlie the propagation of polarity signals along the future polarity axis. The atypical cadherins Fat and Dachsous, and the Golgi protein Four-jointed interpret or establish a directionality cue in order to regulate the movement of the PCP

proteins (Yang, Axelrod et al. 2002; Ma, Yang et al. 2003; Simon 2004). The polarizing signal by Fat, Dachsous, and Four-jointed is amplified and reinforced by a feedback loop between cells to cause asymmetric accumulation of PCP proteins and coordinates cellular polarity across the wing epithelium (Strutt 2001; Tree, Ma et al. 2002; Amonlirdviman, Khare et al. 2005).

Vertebrate PCP Signaling

Vertebrate homologs of the core PCP genes have been identified and these core PCP gene homologs have been shown to be involved in convergent extension (Wallingford, Rowning et al. 2000; Wallingford and Harland 2002), oriented cell division (Gong, Mo et al. 2004), epithelial planar cell polarity (Montcouquiol, Rachel et al. 2003; Devenport, Fuchs et al. 2008) (Figure 1.2). Core PCP proteins asymmetrically localize across the epithelium and this asymmetric localization allows cells within the tissue to orient themselves coordinately along the PCP axis ((Jones and Chen 2007; Seifert and Mlodzik 2007). Mutations in any one core PCP protein cause the disruption of the asymmetric localization of other core PCP proteins within the tissue in addition to the disruption of PCP (Vinson and Adler 1987; Adler, Taylor et al. 2000; Wang, Guo et al. 2006; Deans, Antic et al. 2007).

PCP proteins regulate hair cell polarity in vertebrates in a similar manner to regulation of polarity of hair bristles on *Drosophila* wing (Montcouquiol, Rachel et al. 2003; Wang, Mark et al. 2005; Wang, Guo et al. 2006). Patterning events

other than subcellular localization of PCP proteins appear to determine the orientation of hair cells across the line of reversal. The subcellular localization of PCP proteins Pk2 and Fzd6 have been shown to remain constant across the line of reversal and Pk2 is asymmetrically distributed before bundle morphogenesis at embryonic day 14.5 (Deans, Antic et al. 2007). This indicates that the PCP signaling model for vertebrates is incomplete. It is still unknown when the PCP complex acts in vertebrates. The relative localization of PCP proteins to stereocilia bundles is still unknown for many PCP proteins in the vestibular organs.

Despite the different functions and appearance of the sensory organs of the inner ear, all are composed of an epithelium of mechanosensory hair cells interspersed by supporting cells and all have a distinct PCP. The organ of Corti runs along the entire length of the cochlea and is the mammalian sensory organ for audition. In the rat organ of Corti, Vangl2 begins as a uniform distribution in hair cells and becomes asymmetrically localized to the medial edge of the hair cells (opposite the kinocilium) with Fzd3 (Montcouquiol, Sans et al. 2006).

Ciliary Genes and Relative Centriole Orientation in Vertebrate PCP Regulation

Normally, each cell consists of a pair of centrioles, the mother and the daughter centriole. Centrioles are microtubule organization center of the cell. In a non-dividing, differentiated cell, a daughter centriole localizes to the periphery

while the mother centriole forms the basal body, the root for the cilium. Cilia were first implicated in PCP in a study showing phenotypes associated with PCP mutants (open eyelids, neural tube defects and disrupted cochlear stereociliary bundles) in mice mutant for Bardet-Biedl syndrome ciliary genes (Ross, May-Simera et al. 2005). Ciliary proteins such as Intraflagellar transport protein 88 (Ift88) are known to act in parallel with PCP genes for the regulation of PCP (Jones, Roper et al. 2008). In particular, basal body location correlates with the orientation of cells, and appears to determine the intrinsic polarity of each cell in the cochlea (Jones et al., 2008).

Development of the Utricle and Saccule

Scanning electron microscopy has shown that microvilli and a primary cilium are present on the surface of the utricle between the E11.5 and E12.5 (Takumida and Harada 1984). Short kinocilium and short stereocilia that are about equal in height appear at E13.5 (Takumida and Harada 1984). The polarity of the kinocilium is first detectable with SEM after E16.5 (Takumida and Harada 1984). Phalloidin and acetylated tubulin staining shows that kinocilia are no longer localized to the center of the hair cells after E14.5 (Deans, Antic et al. 2007). At E17.5, hair cells near the line of reversal or “stiola” are mature while immature hair cells are still discernable at the periphery of the utricle(Takumida and Harada 1984). Immature hair cells can be distinguished by their smaller

apical surface area shorter stereocilia relative to mature hair cells (Takumida and Harada 1984; Deans, Antic et al. 2007).

Stereocilia bundles of vestibular mechanosensory hair cells of the utricle and saccule of the inner ear are oriented coordinately to facilitate linear movement detection. Stereocilia bundles consist of a staircase-pattern of microvilli with dense cross-linked actin filaments, the stereocilia, with the tallest stereocilia adjacent to a tubulin-based kinocilium, a primary cilium. Movement of the bundle toward the kinocilium generates an excitatory response and movement away from the kinocilium are thought to cause an inhibitory response (Corey 2003). Hair cells can thus be said to have a polarity determined by the functionally polarized kinocilium and it is this polarization of the kinocilium that makes coordinated orientation of the hair cells in addition to the intrinsic polarity of the stereocilia bundle association with the kinocilium critical for perception of movement in the vestibule and hearing in the organ of Corti.

Furthermore, the sensory hair cells in the saccule and utricle show reverse polarity along a line of polarity reversal, providing a unique opportunity to explore molecular mechanisms for directional control during PCP signaling. The two populations of hair cells across the line of reversal are oriented with kinocilia toward the line of reversal in the utricle and away from the line of reversal in the saccule (Figure 1.3). These two populations of hair cells across the line of reversal in utricle and saccule generate complementary excitatory and inhibitory responses. This may enhance the linear movement perception. The change in

orientation across the line of reversal of the otherwise coordinately polarized hair cells presents a challenge for the current model of planar cell polarity signaling and represents a complex development phenomenon.

Outstanding Issues in Vertebrate PCP Signaling

An instructive global cue has yet to be found for PCP signaling. In addition, it is unknown whether PCP protein localization consistently correlates with hair cell orientation in all vertebrate epithelia as it does in the organ of Corti, and whether this pattern is the same in all vertebrate epithelia.

The line of reversal in the utricle and saccule provides a model for complex planar cell polarization during mammalian development. With two populations of hair cells oriented oppositely on the same epithelium, the utricle and saccule offers a unique opportunity to examine PCP signaling in vertebrates. The line of reversal in the vestibular maculae may provide greater insight into the mechanisms of PCP than the enrichment pattern of PCP proteins in the organ of Corti since all of the hair cells of the organ of Corti are oriented in the same direction along the PCP axis of the epithelium.

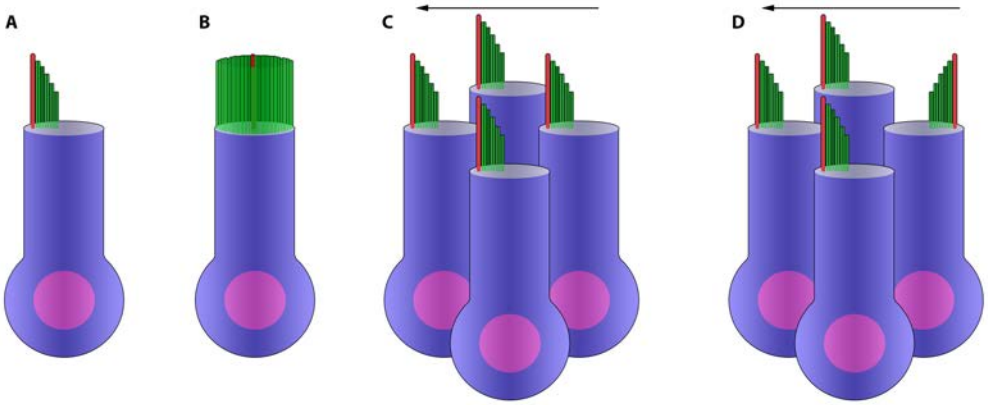
The enrichment pattern of core PCP protein Pk2 has been shown not to change across the line of reversal (Deans, Antic et al. 2007), suggesting that the model extrapolated from the organ of Corti enrichment patterns may be an incomplete one. This reveals that PCP protein localization does not necessarily correspond to cell orientation as was previously assumed. PCP proteins may be

acting in a more permissive capacity than instructive capacity, localization of a subset of PCP proteins may be instructive, or there is an additional mechanism that interprets the PCP protein localization differently on either side of the line of reversal. This is not necessarily an exhaustive list of potential mechanisms at play. In order to determine how widespread the phenomenon observed with Pk2 is in the utricle and saccule, the subcellular localization of Vangl2 and Fzd3 was determined.

In this study, we will first characterize the morphogenetic process to delineate the morphological events leading to the establishment of PCP in the vestibule. Then, with this developmental framework established, we will test the hypothesis that polarization of the kinocilium and other components of the vertebrate PCP pathway directs the establishment of PCP in the vestibular organs.

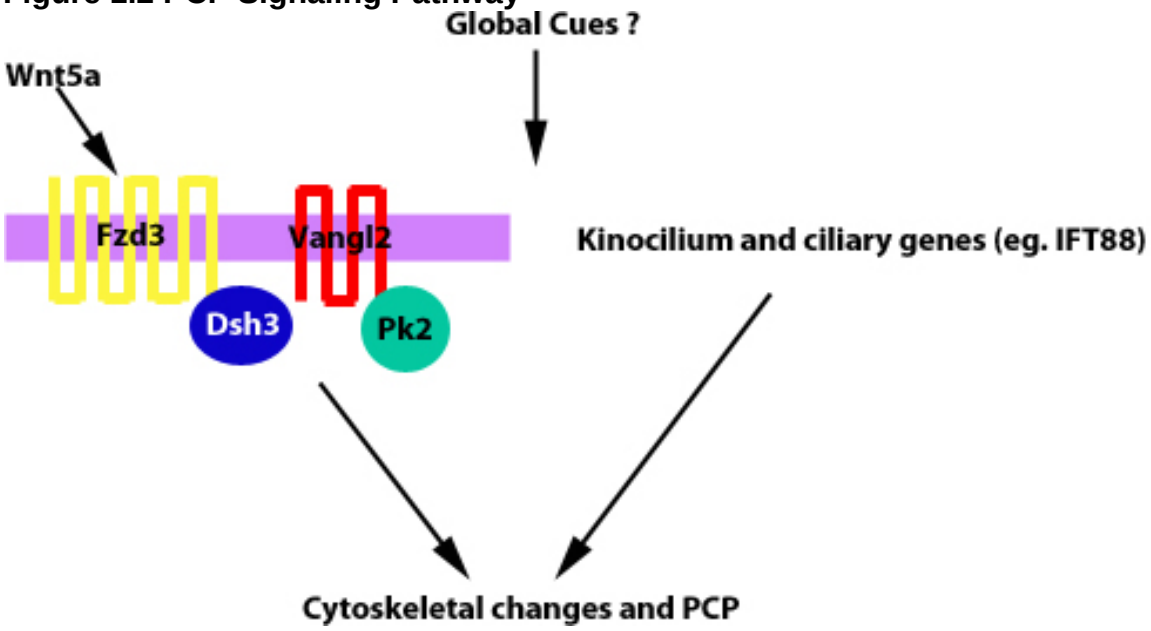
We will provide evidence to support the claim that Vangl2 and Fzd3 show polar distribution in the vestibular maculae and do not change their asymmetric localization patterns across the line of reversal. Additional mechanisms such as the relative basal body orientations may be an effector of PCP across the line of reversal.

Figure 1.1 Intrinsic and Planar Cellular Polarity



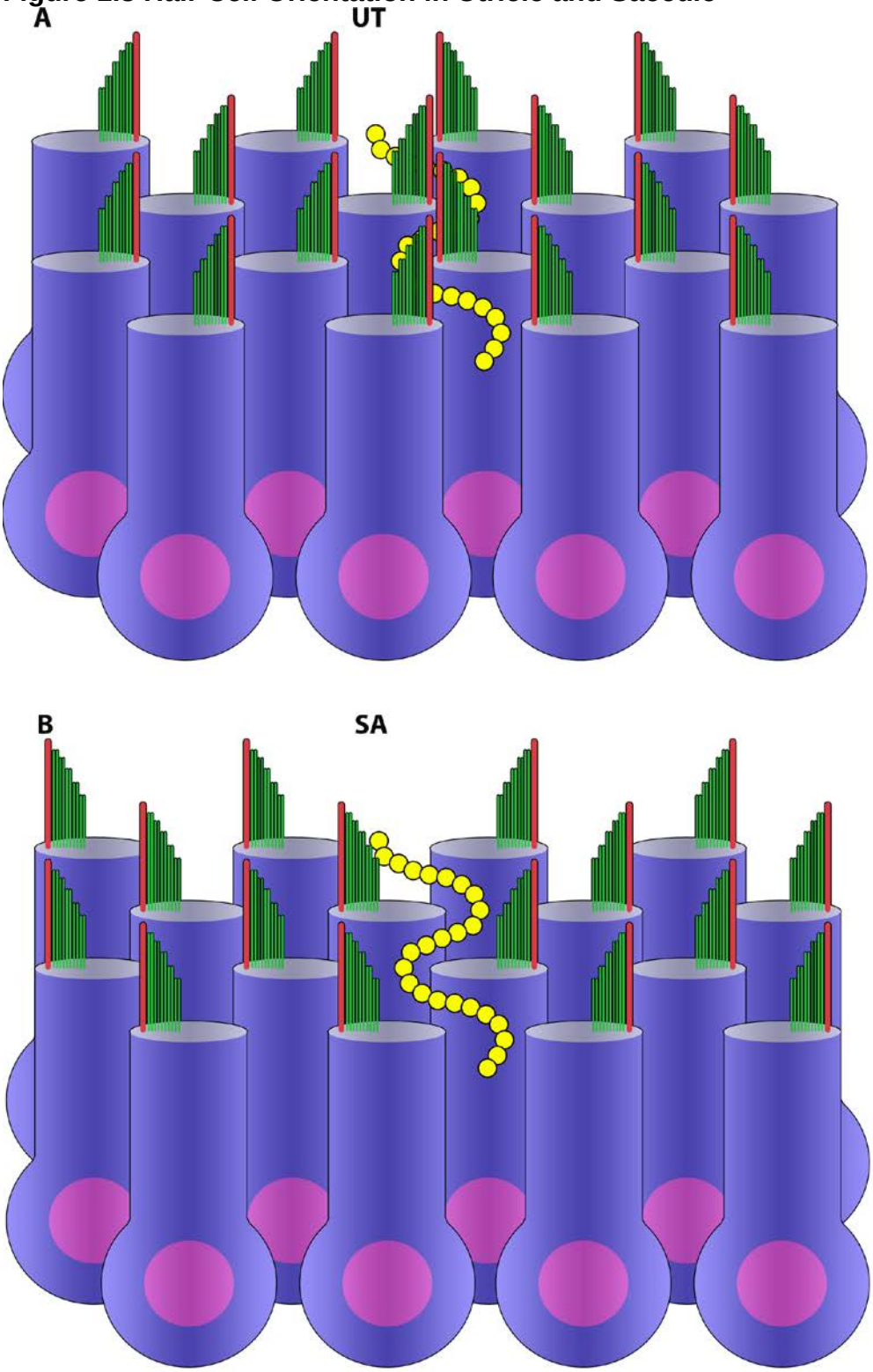
A) Single hair cell with proper intrinsic polarity with kinocilium (red) localized to periphery of the hair cell's apical surface and staircase of stereocilia (green) localized with tallest closest to the kinocilium.
B) Single hair cell with a form of disrupted intrinsic polarity with kinocilium localized to the center of the hair cell's apical surface surrounded by a ring of stereocilia at the periphery.
C) Four cells with both proper intrinsic polarity and planar cell polarity. The planar cell polarity axis is indicated with a black arrow.
D) Four cells with proper intrinsic polarity, but with one cell displaying disrupted planar cell polarity. The planar cell polarity axis is indicated with a black arrow.

Figure 1.2 PCP Signaling Pathway



The vertebrate PCP pathway includes Fzd3, Dsh3, Vangl2, Pk2, and other proteins. Fzd3 is a seven-pass transmembrane protein that responds to noncanonical Wnt signaling such as Wnt5a. Dsh3 is a cytosolic protein that interacts with the cytoplasmic C terminal tail of Fzd3. Vangl2 is a four-pass transmembrane protein that interacts with cytoplasmic Pk2. The kinocilium and ciliary genes such as IFT88 are also involved in establishment of PCP. Flamingo, diego, four-jointed, dachsous, bazooka, and other proteins are excluded from this diagram.

Figure 1.3 Hair Cell Orientation in Utricle and Saccule



(A) The hair cells of the utricle are oriented toward the line of reversal. The kinocilium (red) determines the orientation of the hair cell. Stereocilia are represented in green. The yellow dots represent the line of reversal, which is shown for conception purposes, and is not visible as a physical structure in the epithelium.
(B) The hair cells of the saccule are oriented away from the line of reversal. The kinocilium (red) determines the orientation of the hair cell. Stereocilia are represented in green. The yellow dots represent the line of reversal, which is shown for conception purposes, and is not visible as a physical structure in the epithelium.

Chapter 2 Development of the utricular and saccular maculae of mouse

Introduction

The vestibular maculae have distinct PCP as described in the last chapter. In order to better understand and interpret the utricle and saccule, and thus their respective lines of reversal, as models for planar cell polarity, developmental time points should be established for the morphological events leading to the patterning of the sensory epithelia. Neither these morphological events, nor the establishment of PCP are well characterized. Characterizing the WT vestibular development first provided a background for interpreting the PCP protein localization. This characterization will be very important for the future analysis of mice mutant for known or suspected PCP genes or genes suspected to interact with known PCP genes, such as ciliary genes.

Results

The saccule and utricle grow in size and change shape during development

I used phalloidin to stain the cortical actin cytoskeleton and spectrin as a marker for the fununcule to reveal when the kinocilia polarize from the center to the periphery of the cell. Phalloidin staining reveals the hair cell stereocilia bundle morphology and orientation in addition to staining the cuticular plate and outlining the cytoskeleton the hair cells and supporting cells. Fununcule antibody staining with spectrin revealed the orientation of the kinocilia. I analyzed WT E14.5, E15.5 and E18 saccules and utricles for their morphology after staining with fluorescently-conjugated phalloidin and spectrin antibody with a fluorescently-conjugated secondary antibody.

The saccule and utricle increase in surface area over the course of embryonic development (Figures 2.1, 2.2). Bromodeoxyuridine (BRDU) staining has shown that much of the increase in size after E15.5 is unaccompanied by cellular proliferation (Ping Chen and Dong Qian unpublished data).

Wildtype (WT) sensory hair cell morphology and development in mouse utricle and saccule

A small number of kinocilia are still localized to the center of the hair cells at E13.5, but all kinocilia are polarized by E15.5, being relocated to the periphery of the cell (Figure 2.3). The immature hair cells of the developing utricle and

saccule have shorter stereocilia than more mature hair cells of later stage organs (Figures 2.1, 2.2).

WT intrinsic cell polarization and coordinated polarization occur simultaneously

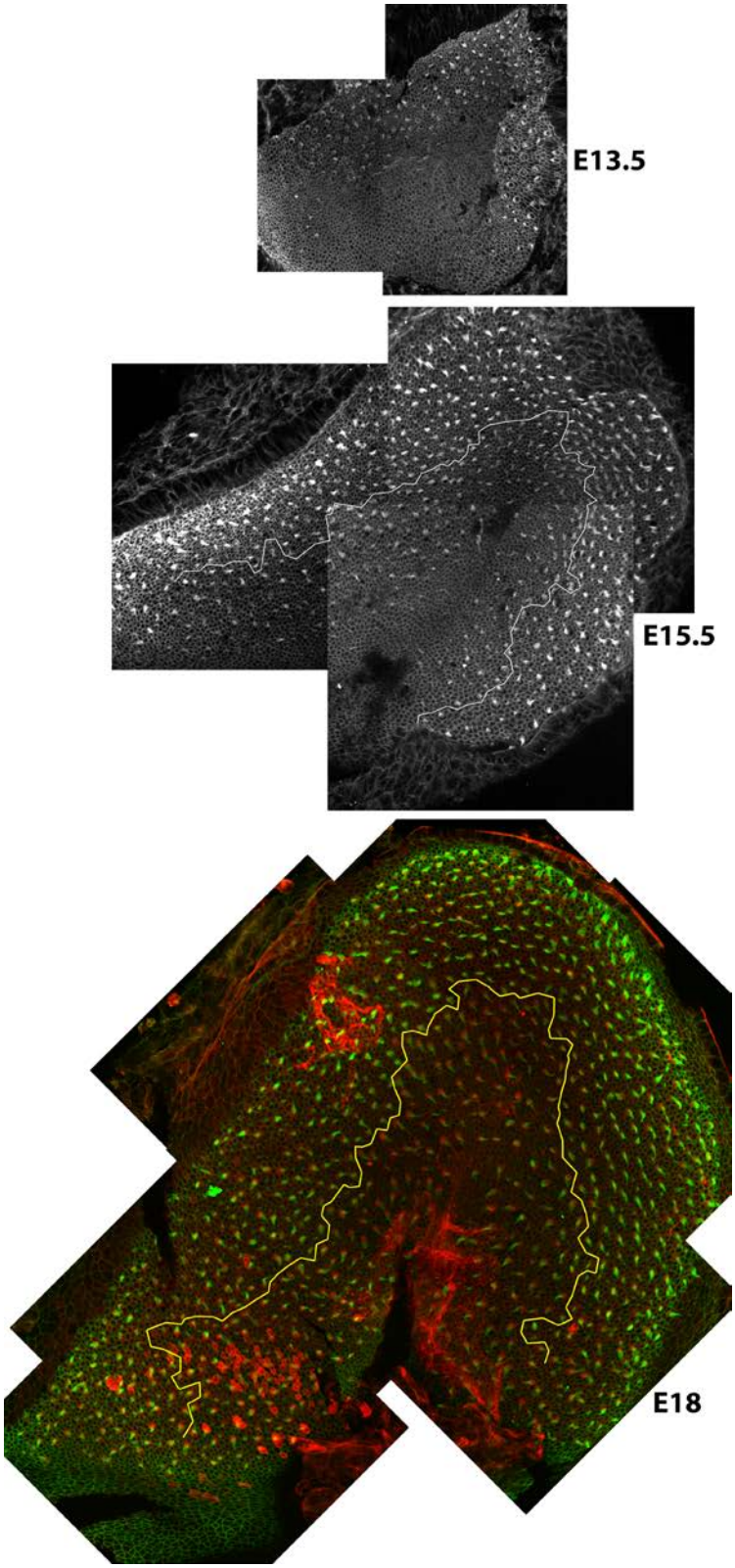
By E13.5 the hair cells have already started to form a line of reversal in saccule and utricle. This line of reversal has a much larger medial region in utricle than the saccule. Rather than intrinisic polarity occurring with stochastic planar polarity followed by reorientation of the hair cells, intrinsic polarization seems to occur simultaneously with planar cell polarization (Figure 2.3). Hair cells with no intrinsic polarity are seen intermixed with hair cells with defined intrinsic and planar cell polarity.

Discussion

Intriguingly, the immature hair cells begin to acquire polarity very early in development. By E13.5 the hair cells have already started to form a line of reversal in saccule and utricle. This line of reversal has a much larger medial region in the E13.5 utricle, whereas in E13.5 saccule, the lateral and medial regions appear approximately equal in surface area as in the later stage saccule. In the utricle, the medial region remains greater than the lateral region throughout development, but not the extreme seen at E13.5 with only a small number of hair cells showing a lateral orientation. Despite these differences in the size, shape, and progression of the development of the line of reversal, it appears that

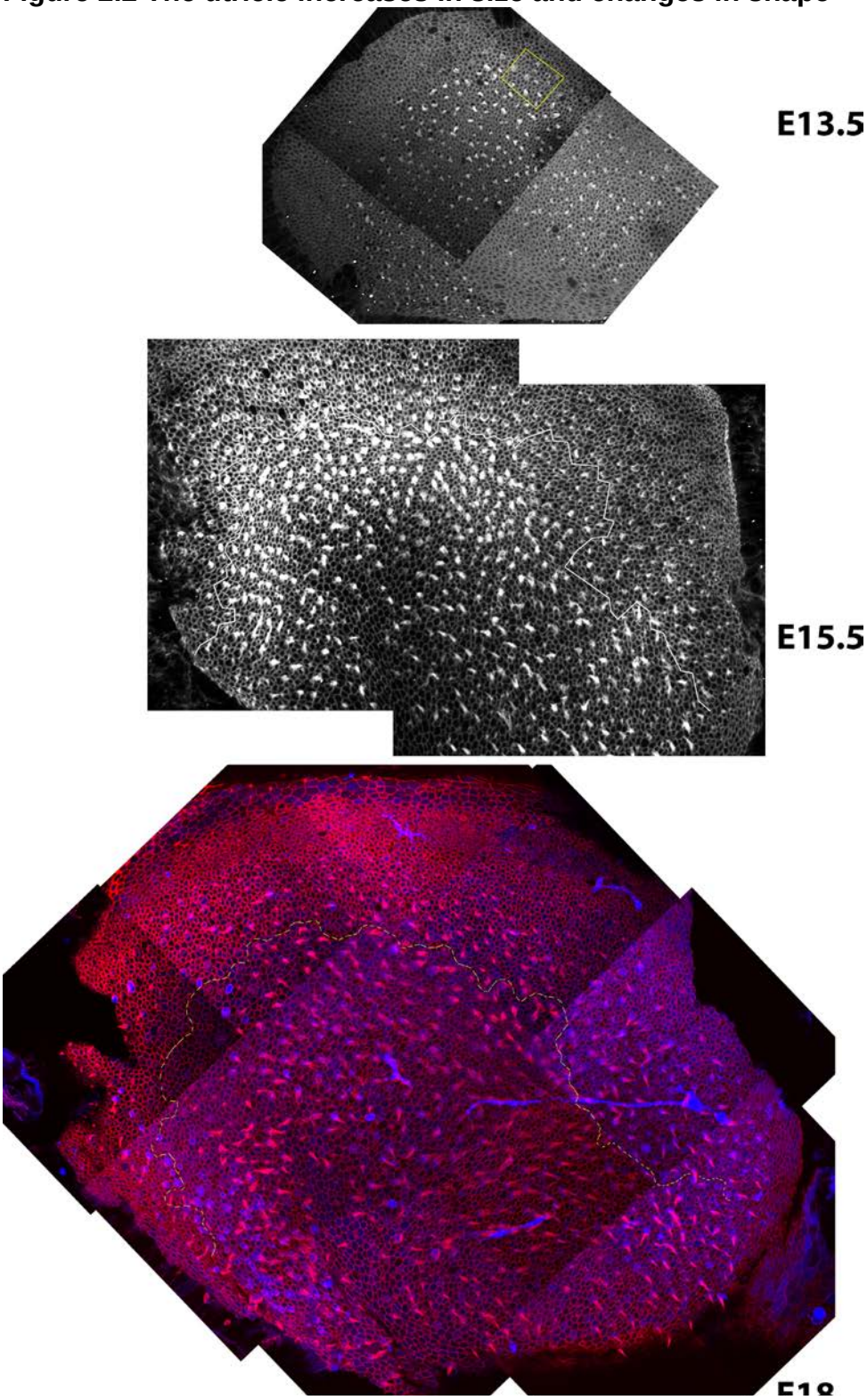
intrinsic and coordinated cellular polarity is established at the same time in these organs.

Figure 2.1 The saccule increases in size and elongates



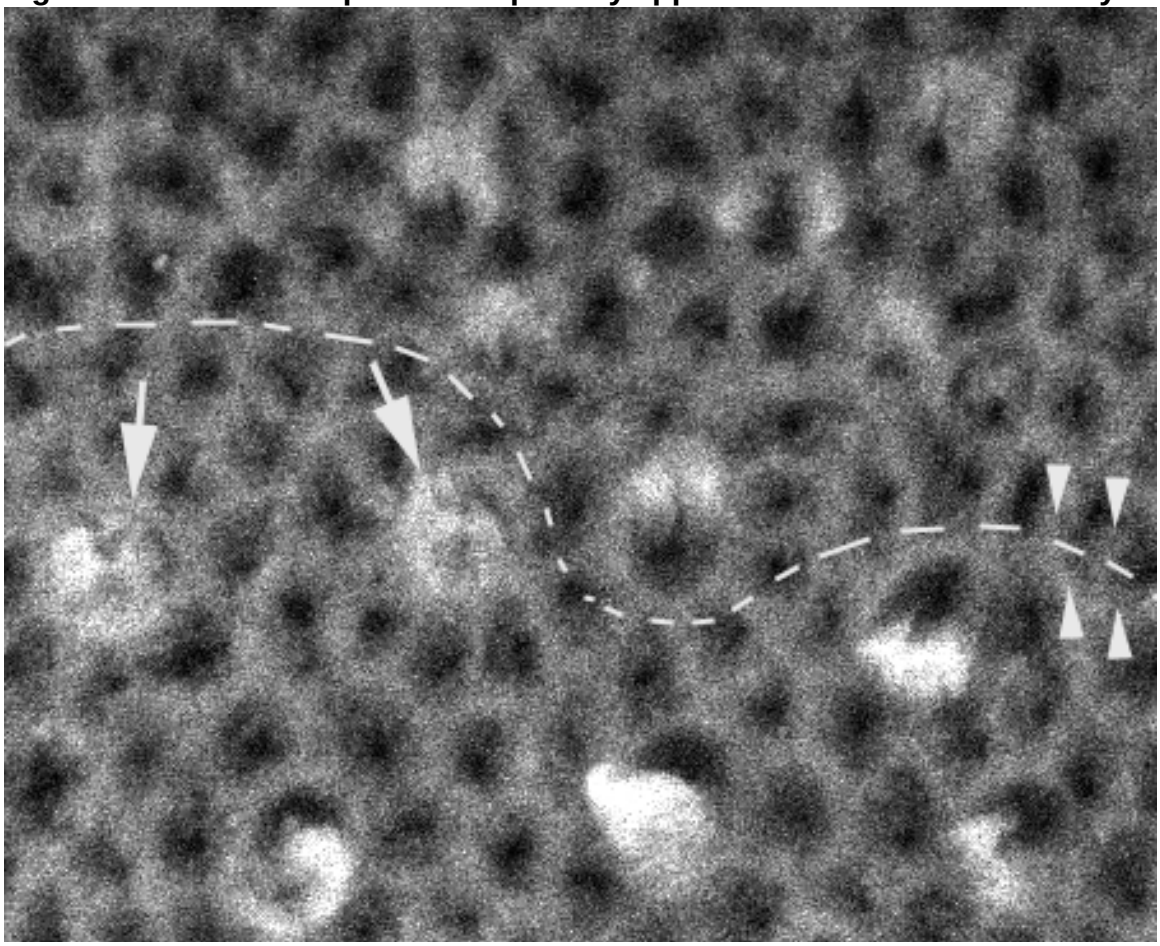
WT E13.5, E15.5, E18 saccules. Gray scale in E13.5 and E15.5 is phalloidin-rhodamine. The saccule increases in size and changes in shape during development, starting as a more round epithelium without ciliated cells in what will be the medial region and becoming a more J-shaped epithelium with ciliated cells covering almost the entire surface. The line of reversal is represented by a white line at E15.5 and a yellow line at E18. n = 5 for all 3 stages.

Figure 2.2 The utricle increases in size and changes in shape



WT E13.5, E15.5, E18 utricles. Gray scale in E13.5 and E15.5 is phalloidin-rhodamine. The utricle increases in size and changes in shape during development, starting as a more round epithelium with far fewer ciliated cells in what will be the medial region and becoming a more 6-shaped ellipsoid epithelium with ciliated cells covering almost the entire surface. The line of reversal is represented by a white line at E15.5 and a yellow line at E18. The yellow box represents the region of the E13.5 utricle which was used for the next figure. n = 5 for all 3 stages.

Figure 2.3 Intrisic and planar cell polarity appear to occur simultaneously



A region from the E13.5 utricle shown in Figure 2.2 marked by the yellow box. Arrows indicate cells that have yet to develop intrinsic polarity. Shortly after sterocilia begin to form, the hair cells are already displaying planar cell polarity and have established a small line of reversal at one edge of the periphery of the organ. Despite the immature sterocilia bundles, the orientation of the hair cells is still clear from the actin-poor outline of the kinocilium along the bundles and the kinocilia roots shown by an actin-poor region of the cuticular plates. Arrowheads indicate the orientation of the hair cells in the two populations of hair cells on either side of the line of reversal. Imaged at 63x.

Chapter 3 Vangl2 is asymmetrically localized in the utricle and saccule

Introduction

The vertebrate PCP pathway includes Vangl2, Fzd3, Pk2, Dsh3, and many other proteins. These proteins are asymmetrically localized in the plane of the epithelium and when the genes for these proteins are mutated, there is a loss of planar cell polarity. Loss of asymmetric localization of one PCP protein leads to the loss of asymmetric localization of the other PCP proteins, suggesting that there may be interdependence for stabilization or trafficking.

Vangl2 is a conserved core PCP protein in vertebrates and homolog of *Drosophila* Vang. A mutation in Vangl2 that spontaneously arose in the Jackson Laboratories called looptail causes a kinked tail in mice that gives the mutation its name as well as spina bifida or cranioarchischisis, deafness, and decreased vestibular function (L. C. Strong 1949; Wilson and Center 1974; Montcouquiol, Rachel et al. 2003). The looptail form of Vangl2 fails to asymmetrically sort in the cell and instead localizes homogenously to the membrane (Montcouquiol, Rachel et al. 2003; Montcouquiol, Sans et al. 2006; Deans, Antic et al. 2007). Mice homozygous for the looptail mutation do not survive gestation, making looptail an embryonic lethal mutation (L. C. Strong 1949; Wilson and Center 1974). In looptail mutants, other PCP proteins are not localized properly either, indicating interdependence between members of the PCP signaling pathway for trafficking

to or stabilization at their proper sites of distribution (Montcouquiol, Sans et al. 2006). In the organ of Corti, Vangl2 is enriched at the medial edge of hair cells opposite kinocilium orientation (Montcouquiol, Rachel et al. 2003; Montcouquiol, Sans et al. 2006).

Results

Vangl2 is asymmetrically localized in the utricle and saccule

Vangl2 is shown here to be asymmetrically localized in both Vangl2-GFP mouse utricle and saccule as well as in viral transfection of eGFP-Vangl2 into cultured WT organs (Figures 3.1-3.5). Polarized distribution of Vangl2 precedes the formation of hair bundles, suggesting PCP proteins act to respond to global cues and establish the PCP axis in the vestibule. The asymmetric localization suggests that it is likely to be involved in PCP patterning in the utricle and saccule in a similar to the organ of Corti. Vangl2-GFP is asymmetrically localized early in development (before E14.5) and this asymmetric localization persists until at least E18.5 (Figures 3.1-3.2).

Vangl2 is enriched along the medial side of hair cell membranes between hair cells and supporting cells

Asymmetric distribution of Vangl2 precedes polarity of kinocilia and formation of mature stereocilia bundles. Vangl2-GFP mouse shows enrichment of Vangl2 at the medial edge of hair cells of both utricle and saccule starting at E14.5 or earlier and persisting through at least E18.5 (Figures 3.1-3.2). At E14.5

the cuticular plate and stereocilia bundles are still immature, so the polarity of individual cells is difficult to assign, but the mediolateral axis is obvious due to asymmetry in the gross morphology of the tissue. It is impossible to determine from this data whether the enrichment at the medial edge of hair cells is due to Vangl2 enrichment at the medial edge of hair cell membranes or the lateral edge of supporting cells.

Vangl2 is enriched along supporting cell membranes parallel to the line of reversal

Vangl2 is enriched at the supporting cell membranes parallel to the line of reversal preferentially relative to the membrane perpendicular to the line of reversal (Figures 3.1-3.2). It is impossible to determine from this data whether the supporting cells have enrichment of Vangl2 on both the medial and lateral regions of their membranes or just at one of these two edges.

Vangl2 is enriched on the medial side of hair cell and supporting cell membranes in single-cell labeling by viral transfection

Achieving a mosaic pattern within the supporting cells and hair cells allowed better resolution of the localization of Vangl2 within a single cell. eGFP-Vangl2 is enriched at the medial edge of membranes of both hair cells and supporting cells in both cultured utricle and saccule (Figures 3.3-3.5). We transfected a lentiviral eGFP-Vangl2 construct into E13.5 saccules and utricles after 1 day in vitro organ culture. These organs were left 6 days in culture

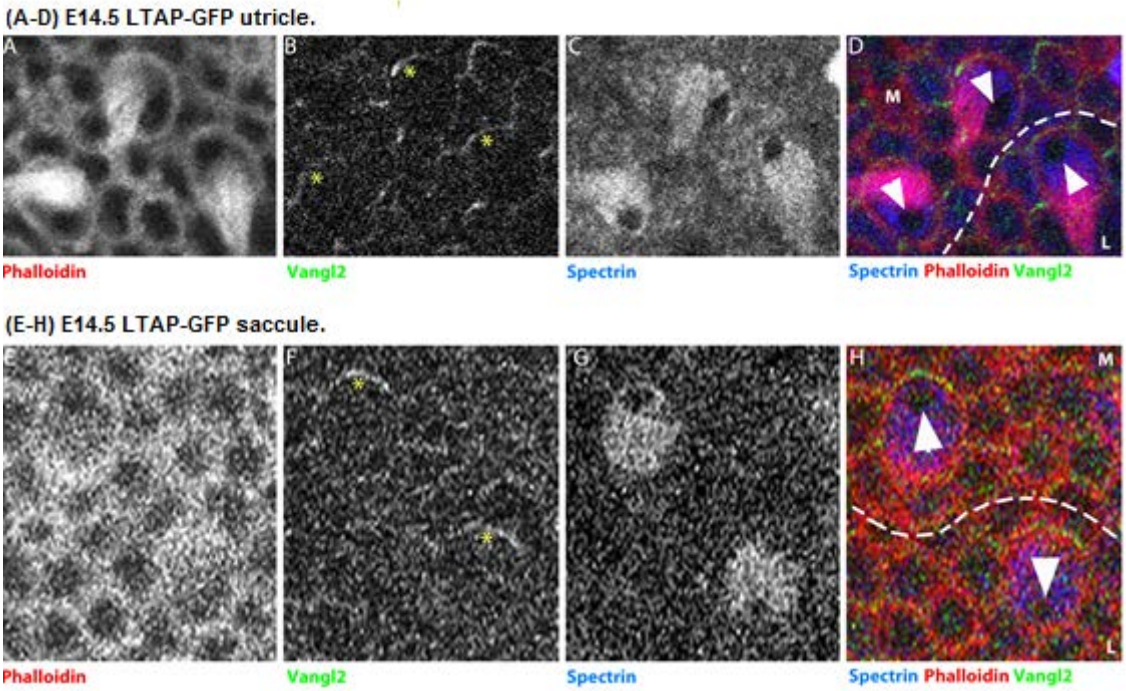
medium for or a total of 7 days in vitro in order to allow high levels of expression of the eGFP-Vangl2 protein.

Discussion

It is intriguing that Vangl2 does not change localization across the line of reversal in utricle and saccule. This indicates that there is differential interpretation of the Vangl2 localization on the medial and lateral edges of these sensory epithelia or that a separate mechanism is determining the polarity of these cells. Vangl2 may thus be a permissive rather than instructive protein in patterning the PCP of these organs. It may be necessary for the correct orientation of the hair cells, but may not encode any information about the PCP axis directionality.

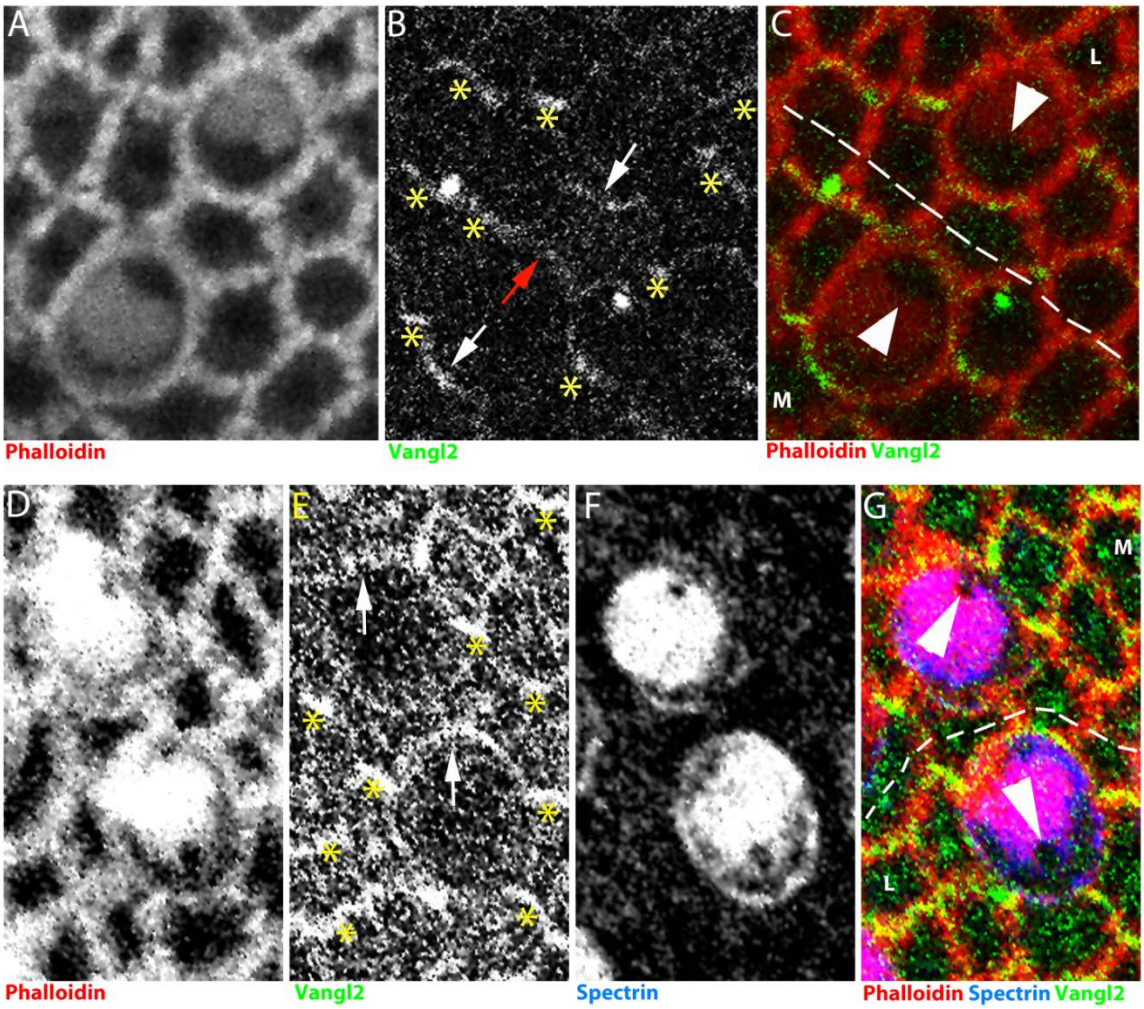
Prickle 2 (Pk2) is also enriched medially in both hair cells and supporting cells of the utricle as shown by electroporation of a Pk2-eGFP construct (Deans, Antic et al. 2007). Frizzled 6 (Fzd6) has also been shown to not change the pattern of enrichment with respect to the hair cells across the line of reversal in utricle, however, the supporting cells have an opposite pattern of enrichment of Fzd6 to the hair cells (Deans, Antic et al. 2007), with medial enrichment in hair cells and lateral enrichment in supporting cells. Fzd3 discussed in the next chapter likely has the same pattern of enrichment as Fzd6.

Figure 3.1 Vangl2 is enriched at the cellular junctions on the medial side of hair cells of the utriclar and saccular maculae early (before E14.5)



Vangl2 accumulates asymmetrically at the junctions between cells of the maculae early in development. (A-D), E14.5 LTAP-GFP utricle. (E-F), E14.5 LTAP-GFP saccule. (A, E), Phalloidin staining outlines the hair cells and supporting cells and stains the cuticular plate of the hair cells, revealing their orientation. (B, F), Vangl2 is enriched at the medial side of the hair cells (yellow asterisks directly beneath) at the border between hair cells and supporting cells. (C, G), Spectrin shows the fununcule and thus orientation of the hair cells with a hole representing the kinocilium. (D, H), Merged phalloidin (red), Vangl2 (green), and spectrin (blue) image. Arrowheads indicate the orientation of hair cells. L and M indicate the lateral and medial sides across the line of reversal (dashed line). In the utricle (D), the hair cells are oriented with the kinocilium toward the line of reversal, while in the saccule (H), hair cells are oriented with the kinocilium away from the line of reversal.

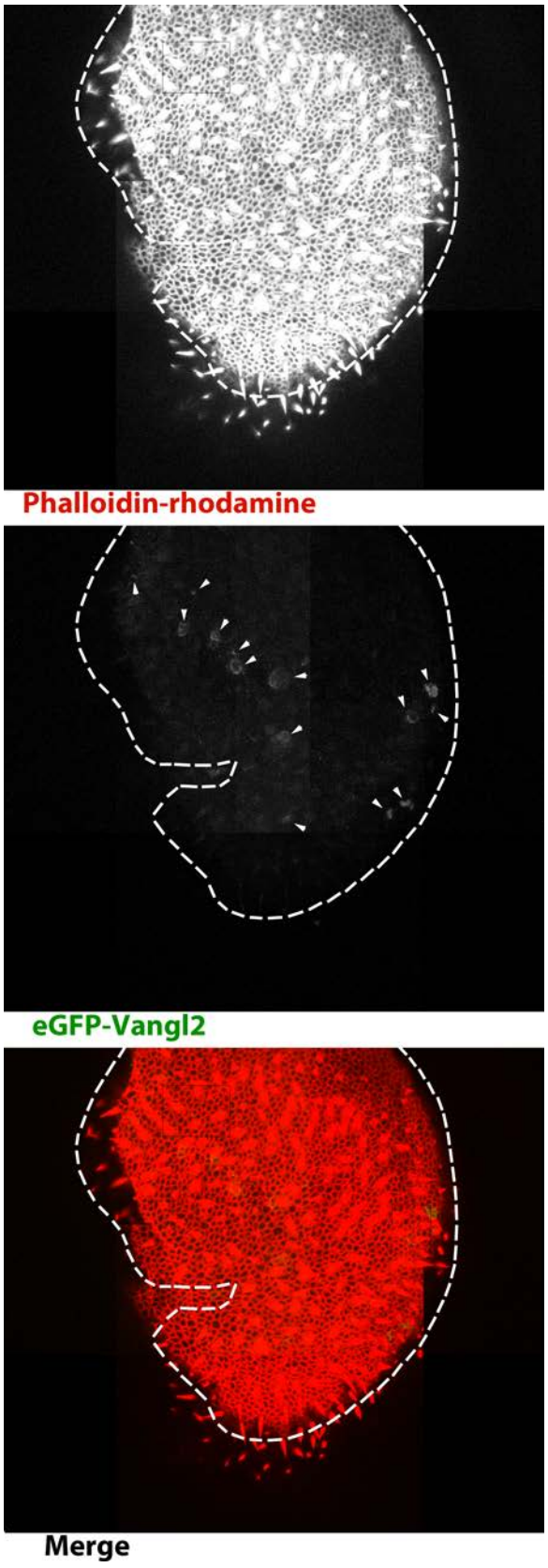
Figure 3.2 Asymmetric localization of Vangl2 persists through development



Vangl2 remains asymmetrically localized during development. (A-C), E18.5 LTAP-GFP utricle. (D-F), E18.5 LTAP-GFP saccule. (A, D), Phalloidin staining outlines the hair cells and supporting cells and stains the cuticular plate of the hair cells, revealing their orientation. (B, E), Vangl2 accumulations at the junctions of supporting cells (yellow asterisks directly underneath) are most pronounced parallel to the line of reversal. Vangl2 is enriched at the medial side of the hair cells (white arrows), but broader, less distinct accumulations of Vangl2 occur frequently at the lateral side of the hair cells (red arrow). (C), Merged phalloidin (red) and Vangl2 (green) image. Arrowheads indicate the orientation of hair cells. L and M indicate the lateral and medial sides across the line of reversal (dashed line). (F), Spectrin shows the fununcule and thus orientation of the hair cells with a hole representing the kinocilium. (G), Merged phalloidin (red), Vangl2

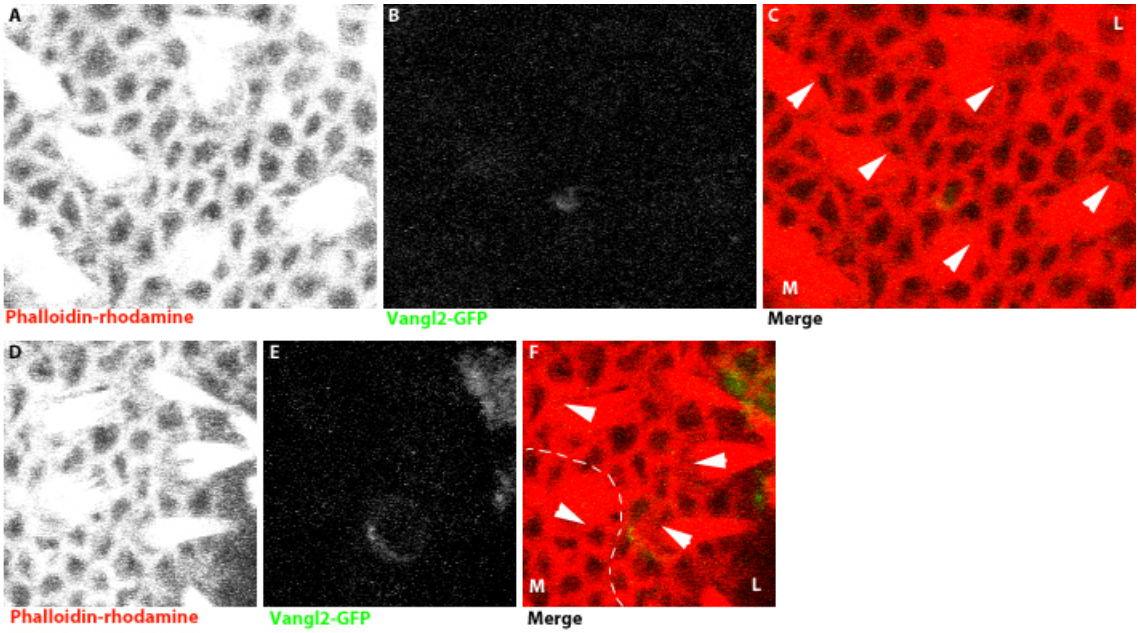
(green), and spectrin (blue) image. Arrowheads indicate the orientation of hair cells. L and M indicate the lateral and medial sides across the line of reversal (dashed line).

Figure 3.3 Transfection Efficiency of eGFP-Vangl2 viral vector



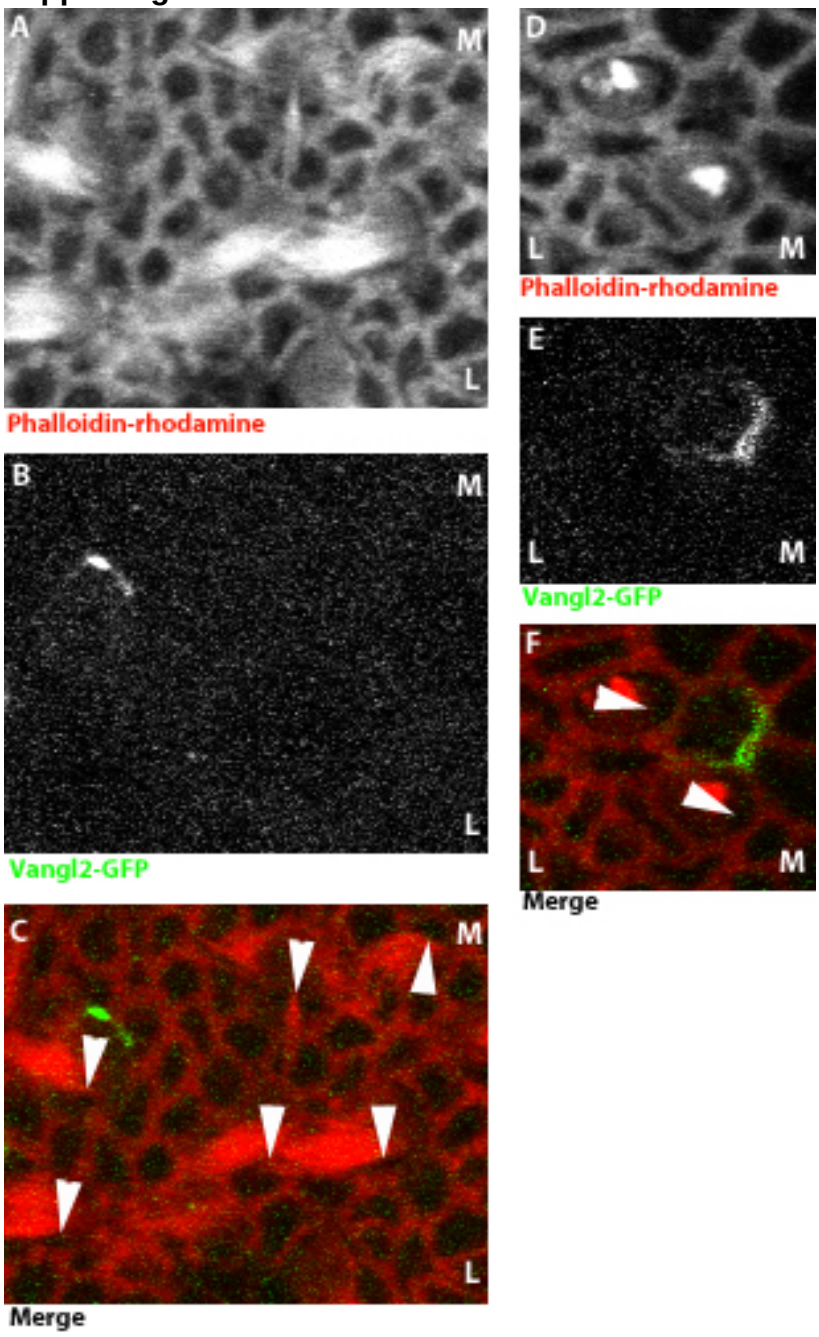
Vangl2 viral vector has low enough transfection efficiency that single-cell analysis of fusion protein expression can be analyzed in the mosaic organ culture. Boxes indicate the location of regions used for the next figure. Tiled 63x images.

Figure 3.4 Single-cell eGFP-Vangl2 labeling of utricle hair cells and supporting cells



E13.5 utricle 7 days in vitro.
A-C) Supporting cell with medial crescent of eGFP-Vangl2 enrichment.
D-F) Hair cell with medial crescent of eGFP-Vangl2 enrichment.
M: medial. L: lateral.

Figure 3.5 Single-cell eGFP-Vangl2 labeling of saccule hair cells and supporting cells



E13.5 saccule 7 days in vitro.
A-C) Supporting cell with medial crescent of eGFP-Vangl2 enrichment.
D-F) Hair cell with medial crescent of eGFP-Vangl2 enrichment.
M: medial. L: lateral.

Chapter 4 Fzd3 is asymmetrically localized in the saccule and utricle

Introduction

Fzd3 is a conserved core PCP transmembrane protein in vertebrates and homolog of *Drosophila* Fzd. Fzd3 and Fzd6 have been shown to asymmetrically localize in the utricle with a bias to localizing parallel to the line of reversal (Wang, Guo et al. 2006). Fzd6-eGFP has been shown to localize to the lateral edge of supporting cell membranes and the medial edge of hair cell membranes in electroporated utricle organs (Deans, Antic et al. 2007).

In order to examine the asymmetric distribution of proteins in the noncanonical Wnt signaling pathway that establish PCP I used confocal microscopy and immunohistochemistry.

Results

Fzd3 is asymmetrically localized in both utricle and saccule.

Fzd3 is enriched primarily parallel to the line of reversal at the membrane of abutting supporting cells as is true of Vangl2-GFP enrichment. As with Vangl2-GFP, Fzd3 enrichment is greater at the contacts between supporting cells than between a hair cell and the surrounding rosette of supporting cells (Figures 4.1-4.2). The asymmetric localization of Fzd3 in utricle and saccule indicates that it is likely that it plays a similar role in the establishment or maintenance of PCP that

it does in the organ of Corti (Wang, Guo et al. 2006). Despite Fzd being the first PCP protein to asymmetrically localize in Drosophila (Vinson, Conover et al. 1989), Fzd3 does not appear to have an asymmetric distribution until late in development (around E18). This could simply be due to poor antibody binding or could be biologically relevant indicating different functions of Fzd3 in the mammalian PCP signaling complex.

Fzd3 colocalizes frequently with Vangl2-GFP on the apical membrane of supporting cells and hair cells.

E18 Vangl2-GFP saccules and utricles were stained antibody stained for Fzd3 in order to reveal the relative localization of the proteins. The Vangl2-GFP signal covers a larger surface of the membrane than Fzd3, but Fzd3 is asymmetrically sorted in a similar pattern to Vangl2-GFP as evidenced by the high degree of colocalization (Figure 4.1-2). Fzd3 and Vangl2 also localize to the same side of hair cells in the organ of Corti (Montcouquiol, Sans et al. 2006; Wang, Guo et al. 2006).

Fzd3 is enriched at the medial edges of hair cells across both sides of the line of reversal in utricle and saccule

Fzd3 is enriched at the medial edges of hair cells across both sides of the line of reversal in utricle and saccule (Figures 4.1-4.2). This is the same pattern observed for Vangl2 and Fzd3 enrichment in the organ of Corti (Montcouquiol, Sans et al. 2006; Wang, Guo et al. 2006). Fzd3 and Vangl2 are on the medial

edges of hair cells and Dsh3 is on the lateral edges of supporting cells in the cochlea (Etheridge, Ray et al. 2008).

The enrichment of Fzd3 medial to hair cells is more distinct apically than the enrichment between abutting supporting cells, while the enrichment between abutting supporting cells is more distinct more basally (Figure 4.3). This suggests that the enrichment of Fzd3 medial to the hair cells may come partially or completely from the hair cell membrane rather than the supporting cell membranes because the apical surfaces of hair cells are higher than the apical surfaces of supporting cells.

Discussion

It appears that Fzd3 enrichment does not change across the line of reversal and thus does not correspond to hair cell orientation in the utricle and saccule. Thus, like Vangl2, it seems that Fzd3 is either interpreted differentially by some additional mechanism on either side of the line of reversal or that Fzd3 does not encode any directional information with respect to the PCP axis (i.e. medial vs lateral).

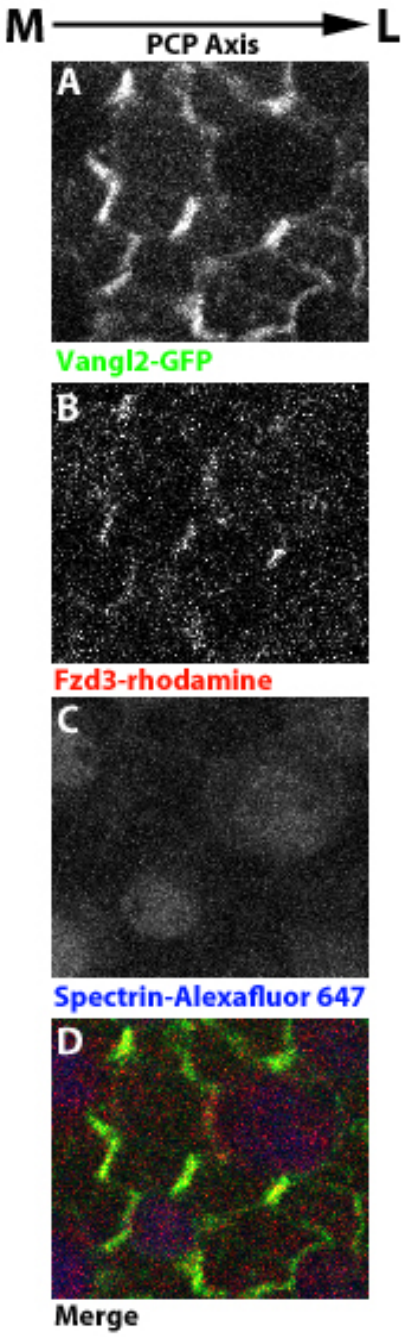
The most apical Fzd3 localization appears to be associated with the hair cells, indicating that the hair cells may be contributing to at least part of the Fzd3 enrichment at their medial edges. Even if this is true, whether Fzd3 enrichment medial to hair cells is from hair cells exclusively or from both hair cells and

supporting cells is not discernable with these confocal microscopy techniques at these resolutions.

Fzd3 appears to colocalize with Vangl2 as it does in the cochlea. It could be that the coloclization pattern with Vangl2 represents the same pattern of enrichment of Fzd3, but other possibilities cannot be ruled out without employing different techniques such as single-cell labeling.

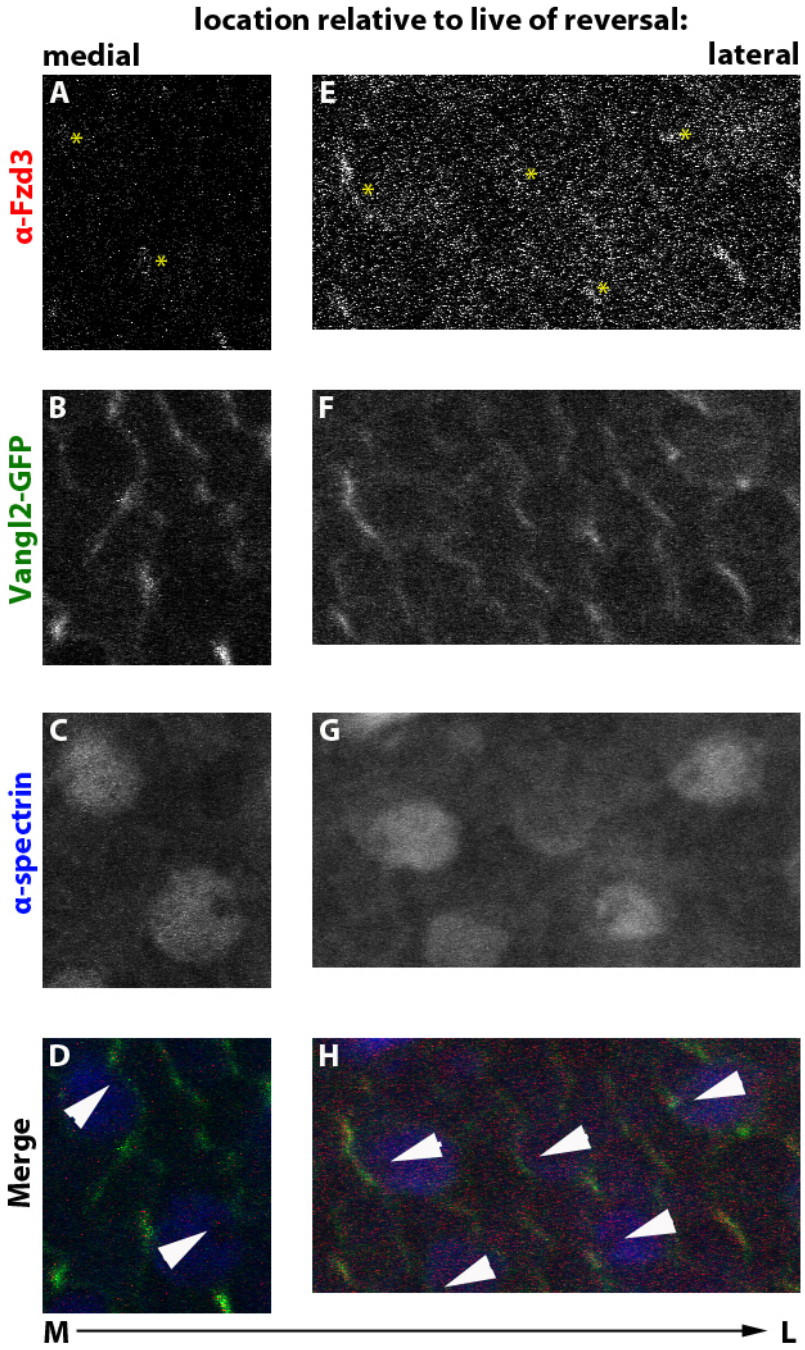
Fzd3 is asymmetrically sorted in a similar pattern to Vangl2-GFP as evidenced by the high degree of colocalization. This is the same pattern observed for Vangl2 and Fzd3 enrichment in the cochlea. Fzd3 and Vangl2 are on the medial edges of hair cells and Dsh3 is on the lateral edges of supporting cells in the cochlea. It is worth noting that the Dsh3 pattern does not match the Fzd3 pattern even though the transmembrane protein Fzd recruits cytoplasmic Dsh in Drosophila. This makes Dsh3 the only PCP protein known to localize to the same side as of the hair cells as the kinocilia in cochlea as well. This makes Dsh3 an attractive candidate for instructive signaling in the utricle and saccule across the line of reversal.

Figure 4.1 Fzd3 is enriched at the medial edge of hair cells in the saccule



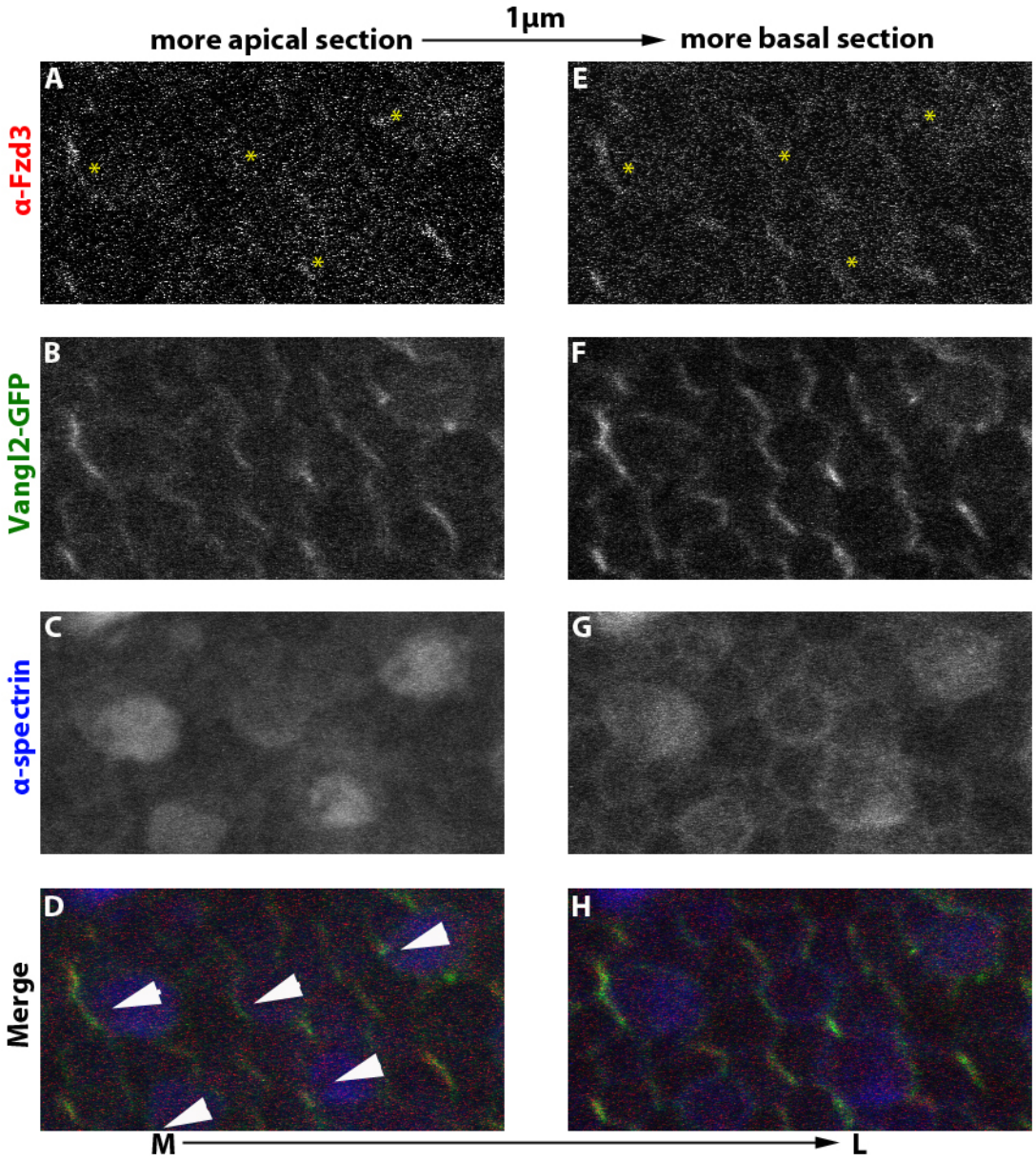
A-D) E18 Vangl2-GFP saccule with Fzd3 enrichment on the medial side of hair cell membranes showing a high degree of colocalization with Vangl2-GFP.

Figure 4.2 Fzd3 is enriched on the medial edge of hair cells in the utricle



E18 Vangl2-GFP utricle.
A-D) A region of the utricle medial to the line of reversal.
E-H) A region of the utricle lateral to the line of reversal.
Asterisks indicate the intracellular side of Fzd3 enrichments at the medial edges of hair cells. Arrowheads indicate the orientation of the hair cells.

Figure 4.3 Fzd3 distribution changes with respect to the apicobasal axis



E18 Vangl2-GFP utricle.

A-D) A region of the utricle lateral to the line of reversal.

E-H) The same region of the utricle 1µm more basal. The Fzd3 enrichments at the edges of hair cells are less distinct, but the Fzd3 enrichments at the regions of two abutting supporting cells are more distinct.

Asterisks indicate the intracellular side of Fzd3 enrichments at the medial edges of hair cells. Arrowheads indicate the orientation of the hair cells.

Chapter 5 Overall Conclusions and Future Directions

The line of reversal in the utricle and saccule provides a model for complex planar cell polarization during mammalian development. This system may provide greater insight into the mechanisms of PCP than the enrichment pattern of PCP proteins in the organ of Corti. PCP proteins may be required for other directional cues to be interpreted, indicating that PCP proteins may be acting in a more permissive capacity than instructive capacity. One possibility is that the PCP proteins are asymmetrically localized in response to some global cue(s) across the tissue and are required for the establishment and/or maintenance of the coordinated cellular orientation. The enrichment pattern of core PCP protein Pk2 has been shown not to change across the line of reversal (Deans, Antic et al. 2007), suggesting that the model extrapolated from the organ of Corti enrichment patterns may be an incomplete one. This reveals that PCP protein localization does not necessarily correspond to cell orientation as was previously assumed.

In order to determine whether other PCP proteins change their asymmetric sorting pattern across the line of reversal, I analyzed Vangl2 and Fzd3 enrichment patterns after characterization of WT morphological changes during development. To achieve this, I imaged Vangl2-GFP transgenic mouse organs and immunostained WT and Vangl2-GFP mouse organs with a Fzd3 antibody. In order to examine single-cell localizations of Vangl2, I used a viral vector to transfect WT organs with eGFP-Vangl2 fusion protein. I observed

Vangl2 to be medially enriched in both hair cells and supporting cells. Fzd3 colocalized with Vangl2, but we cannot establish absolute localization without some form of labeling that gives single-cell resolution.

The results shown here support the observation that there is an apical region of vertebrate epithelial cells that is enriched for PCP signaling molecules and that the enrichment of PCP signaling molecules in this region is important for PCP establishment (Usui, Shima et al. 1999; Djiane, Yogev et al. 2005). Vangl2 and Fzd3 both appear to be enriched at the apical surface of hair cells and supporting cells and are asymmetrically localized within the plane of the epithelium in utricle and saccule. Furthermore, the most apical region of Vangl2 and Fzd3 enrichment in utricle and saccule appears to be along the medial edges of hair cells. Viral transfection of eGFP-Vangl2 showed a medial enrichment of eGFP-Vangl2 in both supporting cells and hair cells. This pattern of asymmetric sorting of Vangl2 is one possible explanation for the fainter lateral accumulations of Vangl2-GFP frequently observed opposite the medial enrichments of Vangl2-GFP. Previous work electroporating eGFP fusion protein plasmid contructs into utricle tissue has shown that Pk2 is enriched on the medial side of both hair cells and supporting cells while Fzd6 is enriched medially in hair cells and laterally in supporting cells. Presumably this is the same for the saccule. The localization of Vangl2, Fzd3, Fzd6, and Pk2 is diagrammed in Figure 5.1.

Future directions

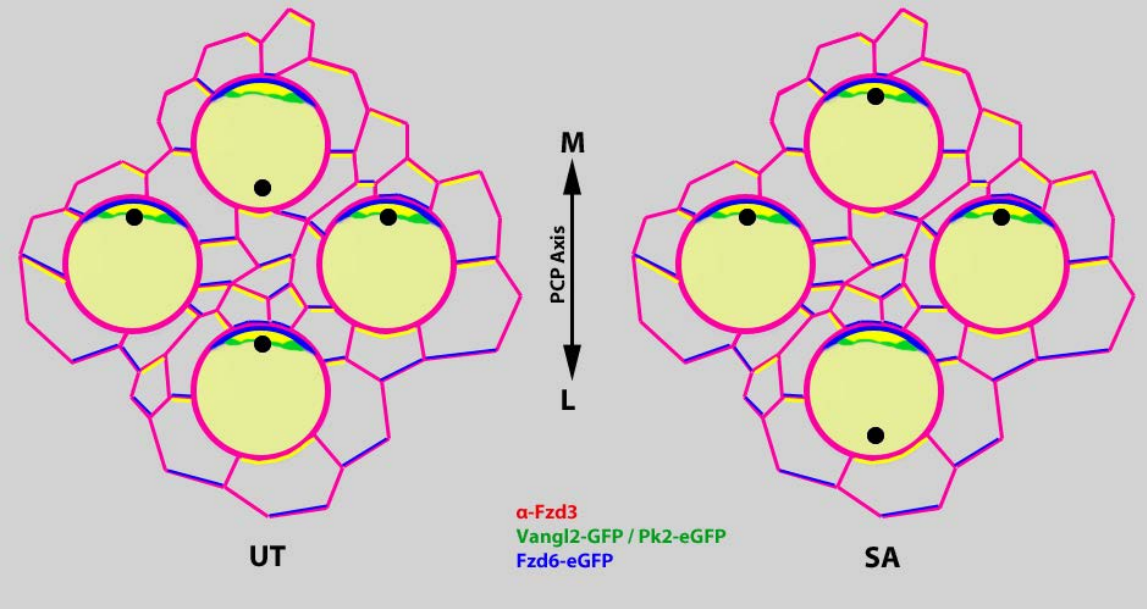
It is not possible from the techniques used to determine whether this Fzd3 enrichment is from the medial or lateral edges of supporting cells causing the enrichment at the edges of two abutting supporting cells. It is also not possible from these techniques to determine whether the Fzd3 enrichment at the medial edges of hair cells is a medial accumulation at hair cell membranes, a lateral accumulation at the supporting cell membranes, or both. Generation of a mouse line with a mosaic epithelium with a differentially expressed marker, electroporation, or viral transfection of a fluorescent fusion protein could be used to overcome this limitation. A Fzd3/6-GFP or Fzd3/6-Cherry plasmid could be used to determine the single-cell distribution of Fzd3/6 within the saccule and utricle. We have generated a Fzd6-Cherry plasmid from a Fzd6-GFP that was a gift from the Goodrich lab. This plasmid could be electroporated into cells or used to generate a viral vector similar to the eGFP-Vangl2 viral vector used in this study.

To assess whether basal body polarity acts as a polarity determinant for individual cells, the polarity of basal bodies across the line of polarity reversal in the maculae will be determined. Determining whether basal body orientation is different between mutant and WT vestibular will allow us to study the dependence on basal body orientation for intrinsic cell polarity. Polaris, also known as IFT88, is a protein involved in ciliogenesis, and has been shown to affect the intrinsic cell polarity by disrupting the polarity of the stereociliary

bundles, but does not affect the distribution of the core PCP proteins (Jones, Roper et al. 2008). Determining PCP disruption in Arl13b mutants using the (A GTPase that localizes to the kinocilia) Arl13b$^{flox/flox}$ x Pax2Cre or Cre^{ESR1} (tamoxifen inducible lines) will establish whether other ciliary genes have a similar phenotype which implies that ciliogenesis and PCP signaling act in parallel pathways.

Localization of another core-PCP homolog in mice, Dishevelled 3 (Dsh3/Dvl3), could be determined using a transgenic mouse line expressing Dsh3-GFP fusion protein. There is not a good antibody for the Dishevelled homologs in mouse, but a Dsh3-eYFP mouse mutant has shown that Dsh3 is asymmetrically localized in the utricle and organ of Corti (Etheridge, Ray et al. 2008). Dishevelled is an attractive PCP protein for possible differential localization across the line of reversal. Unlike Fzd3 and Vangl2, Dsh3 localizes to the same side of hair cells as the kinocilia (lateral) in the organ of Corti (Montcouquiol, Sans et al. 2006; Wang, Guo et al. 2006; Etheridge, Ray et al. 2008). Dsh3 associates with basal bodies and is essential for their docking at the apical plasma membrane and for coordinated cilia beating in Xenopus (Park, Mitchell et al. 2008).

Figure 5.1 Fzd3 and Vangl2 localization in utricle and saccule



Vangl2 is enriched on the medial edge of hair cell membranes and supporting cells membranes in both utricle and saccule. No single-cell Fzd3 labeling is available to distinguish the orientation of Fzd3. Colocalization of Fzd3 to Vangl2 is represented in yellow as shown by the results of this study, but it is possible that Fzd3 accumulations may instead be on the lateral edges of hair cells and supporting cells or other combinations of enrichment that would give rise to the same colocalization pattern. Fzd6 is shown in blue. The black circles on the apical surfaces of the hair cells (beige) represent the kinocilia. Magenta is used to outline the hair cells and supporting cells in the same manner that phalloidin staining would.
UT: utricle.
SA: saccule.
M: medial.
L: lateral.

Chapter 6 Materials and Methods

Mouse Strains and animal care

Animal care and use was in accordance with US National Institutes of Health (NIH) guidelines and was approved by the Animal Care and Use Committee of Emory University. Vangl2-GFP (Qian, Jones et al. 2007) and FVB mouse strains were used in this study. The FVB mouse strain is from Jackson Laboratories. For staged embryos, the morning after mating was designated as E0.5.

Viral Transfection of Organ Culture

Emory viral core produced the adenovirus vector once provided with the fusion protein cloned into the pFUGW vector. Organs were first harvested from embryos and dissected in Dulbecco's phosphate buffered saline (Gibco). Organs were then cultured on poly-d-lysine (Sigma) coated plates in media containing 87% Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco), 10% fetal bovine serum (Atlanta Biologicals), 2% B27 (Gibco), and 1% penicillin. Virus was added to the media overnight and then the organs were grown for a number of days in fresh media.

Whole mount analysis of inner ear tissues

Primary antibodies and dies used were Fzd3 (1:50) (Wang, Guo et al. 2006), Spectrin (1:200, Millipore), Alexafluor Phalloidin-488 (1:1000, Invitrogen) and Alexafluor-Phalloidin-rhodamine (1:1000, Invitrogen), Arl13b (1:1,500) (Caspary, Larkins et al. 2007), Gamma-tubulin (1:200, Sigma). Images were acquired with

Zeiss LSM510 and Olympus Fluoroview FV-1000 confocal microscopes using 1µm or less optical Z-sections.

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