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Development of the stereocilia and kinocilia in the mammalian organ of Corti as directed by Rab11a

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Abstract

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The organ of Corti in the mammalian cochlea is precisely designed for its function of auditory perception. Sensory hair cells have apical projections consisting of stereocilia organized into a V-shaped bundle and a kinocilium at the vertex representing the intrinsic polarity of the hair cell. Coordinated polarity of the cell is established through the planar cell polarity pathway. In order for the intrinsic and coordinated polarity of the hair cell to be established, proteins involved in these pathways must be specifically trafficked to the correct cellular compartments. Rab11a is a protein of the recycling endosome also necessary for ciliation that is positioned to play a role in cochlear hair cell development. Using Rab11a conditional knockout mice, we investigated the role that Rab11a plays in the formation and maintenance of the hair cell. In cultured cells we found that Rab11a was necessary for ciliogenesis. However, in the hair cell, Rab11a regulates kinocilia formation through a genetic interaction with IFT88. Stereocilia formation is affected in Rab11a conditional knockouts leading to stereocilia bundled with an altered intrinsic polarity no longer resembling the V-shaped bundle but instead have fragmented bundles. Additionally, Rab11a loss leads to stereocilia degeneration and hearing loss in adult mice. Loss of Rab11a did not alter apical protein trafficking in the cochlea suggesting the malformation of stereocilia was not due to a global loss of apical protein trafficking. We found that expression of dominant negative Rab11a construct in culture altered the trafficking of Usher protein Cadherin23 from the apical domain of the cell to throughout the entire cell suggesting that Rab11a may be responsible for trafficking inter-stereocilia link proteins responsible for stereocilia adhesion. Additionally, the planar cell polarity pathway is regulated downstream of core PCP proteins by Rab11a and Ankrd6, the mammalian homolog of Diego. Together, these data show that Rab11a plays vital roles in hair cell formation and maintenance through its roles in kinocilia formation, regulation of the planar cell polarity pathway, stereocilia formation, and ultimately hearing suggesting that intracellular trafficking in hair cells is a vital part of hair cell development.

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CHAPTER 1: The Inner Ear

The mammalian inner ear is housed within the temporal bone composed of the cochlea responsible for audition and the vestibular system responsible for the sensation of orientation and angular acceleration. The vestibular system consists of semicircular canals containing the anterior, lateral, and posterior cristae detecting angular acceleration and the macular utricle and saccule detecting linear acceleration and gravity (Figure 1.1A). Although the aspects discussed in this dissertation apply specifically to the cochlea, several cellular features and the formation of the mechanosensory apparatus, the stereocilia bundle of the sensory hair cells, apply to the vestibular system as well.

Within the bony labyrinth of the cochlea are three membranous labyrinths, the scala vestibule, the scala media or cochlear duct, and the scala tympani (Figure 1.1B). Within the scala media, atop a flexible basilar membrane, sits the organ of Corti, the sensory organ responsible for audition (Bekesy, 1952). The organ of Corti is composed of three rows of outer hair cells (OHCs) and a row of inner hair cells (IHCs) interdigitated with nonsensory supporting cells (Figure 1.1C-D). The IHCs are the primary cochlear transducers of sound having afferent innervation while the OHCs almost exclusively innervate to efferent neurons and have mobility thought to allow for the ability to distinguish frequency selectivity (LeMasurier and Gillespie, 2005; Zheng et al., 2000a). In humans, each of the 15,000 to 30,000 hair cells (HCs) within the organ of Corti is adorned with a hair bundle consisting of actin-filled stereocilia on the apical surface (Frolenkov et al., 2004). The hair bundle stereocilia form a V, W, or U shape pointed towards the lateral edge of the hair cell (Figure 1.1D). Each stereocilia is graded

in height with the longest of the approximately three rows towards the vertex (El-Amraoui and Petit, 2005). Preceding stereocilia formation, a single true microtubule based cilia termed the kinocilium emerges. The kinocilium ultimately lies at the vertex of the stereocilia bundle emanating from the basal body (Sobkowicz et al., 1995). The kinocilium sitting at the vertex of the stereocilia bundle of each hair cell and the stereotypic arrangement of the stereocilia constitutes the intrinsic polarity of each hair cell while the coordination of the polarity across the organ of Corti keeps every hair cell oriented in the same direction (Figure 1.1D, 1.3A). The precise development of the cochlea and the cochlear hair cells is paramount for the function of the organ of Corti in auditory perception.

Development of the Cochlea

The organ of Corti requires a remarkable number of embryonic signaling events to regulate the precise formation of the highly organized structure that allows for transforming sound into neural signals received by the brain. The morphological features and molecular mechanisms of hearing between the mouse and human cochlea and organ of Corti are remarkably similar. As such, the mouse has been used to decipher molecular pathways needed to form the hearing machinery of the organ of Corti. In addition, studies involving other model systems in invertebrates and vertebrates, particularly *Drosophila* and *Xenopus*, have largely shaped the current understanding of the fundamental biological pathways regulating cochlear development.

Morphological Development of the Organ of Corti

Early in embryonic development, the embryo interprets global patterning signals from across the tissue to influence cellular movements and rearrangements of cells.

These cellular rearrangements shape the body plan and set up the body axes, and give rise to individual organs and tissues of an organism. While the scope of embryogenesis is large and complex, the formation of the cochlea has been well studied and recorded. In mice, at embryonic day 8.5 (E8.5) a small patch of ectoderm near the hindbrain, termed the otic placode, invaginates to form the otic pit (Kikuchi et al., 1988). This otic pit later closes becoming the fluid filled otic vesicle by E10.5 (Kikuchi et al., 1988).The ventral otic vesicle outpockets and elongates at E12.5 to form the cochlear duct containing an epithelial ridge on the duct floor demarking a morphologically and molecularly distinct epithelium destined to become the auditory sensory organ, the organ of Corti (Morsli et al., 1998). Additionally, other regions of the otic vesicles form the fluid filled sacs of the utricle, saccule, and semicircular canals of the vestibular system (Morsli et al., 1998). The epithelial ridge on the floor of the cochlear duct thickens and between E12.5 to E14.5 and the cells in the epithelial ridge withdraw from the cell cycle to form a zone of non-proliferating cells termed the sensory primordium in a gradient from the apex to the base of the cochlea (Kelly and Chen, 2007; Ruben, 1967). The sensory primordium relies on external molecules (Shh, BMP, FGF, Wnts) and activation of genes (transcription factors, notch signaling, fibroblast growth factors, and Math1) to determine the sensory and nonsensory cell lineages and boundaries of the organ of Corti during cellular differentiation (Kelly and Chen, 2007). Tissue morphogenesis continues to occur as the sensory region undergoes convergent extension where the tissue narrows along mediolateral axis to double in length along the apical to basal cochlear axis (Wang et al., 2005). Convergent extension of the cochlear epithelia occurs between E14.5 and E18.5 during which time the cochlea

doubles in length and thins from 4-5 cells thick to 2 cells thick (Chen et al., 2002; Wang et al., 2005).

Cellular differentiation of the cochlear sensory epithelial cells occurs in a gradient throughout the cochlea beginning at the base of the cochlear duct and working its way apically (Lelli et al., 2009; Waguespack et al., 2007). Additionally, the IHCs differentiate before the OHCs on the mediolateral axis (Kelly and Chen, 2007). This graded development underlies the differences in the maturity of sensory hair cells along the mediolateral axis and along the length of the cochlear duct of the organ of Corti observed during development.

Hair Cell Development and Patterning

Concurrent to the morphological events occurring to shape the organ of Corti at the tissue level, the sensory hair cells undergo morphological changes**.** The sensory hair cell of the cochlea is adorned with precisely formed and oriented apical projections necessary for the transduction of sound to a neuronal signal. Below I will discuss the development processes responsible for the development and patterning of the hair cell (Figure 1.2).

Planar Cell Polarity

Planar cell polarity (PCP) refers to the coordinated orientation of cells and cellular components across the plane of a tissue. Body hair orientation, cilia orientation in multiciliated airway cells, and oriented of sensory hair cells are examples of PCP (Klein and Mlodzik, 2005; Vladar et al., 2012). Additionally, major cellular processes in embryonic development including convergent extension, the elongation of a tissue mediolaterally to enable directional movement of cells and intercalations of neighboring

cell layers. Among these processes are gastrulation resulting in the formation of the three germ layers, neurulation or neural tube closure, and the extension of the cochlear duct (Chen et al., 2002; Simons and Mlodzik, 2008; Wallingford, 2012; Wang et al., 2005). Convergent extension of the embryo drives the establishment of the body plan and of individual organs to shape the form for function (Henderson et al., 2018). Defects in genes regulating PCP or the PCP pathway cause short stature, heart diseases, polycystic kidney disease, respiratory diseases and many other conditions displaying the vast role of PCP in development (Fischer and Pontoglio, 2009; Wu et al., 2011; Yates and Dean, 2011).

The sensory organs of the inner ear represent arguably the most distinctive forms of PCP in vertebrates. The organ of Corti in the cochlea consists of four rows of sensory hair cells with a precisely formed apical surface including a kinocilium and a V-shaped stereocilia bundle. The stereocilia bundles of all of the hair cells in the organ of Corti are oriented uniformly, displaying a distinctive form of PCP.

The regulation of many forms of PCP, including the uniform orientation of hair cells in the cochlea is by a conserved genetic pathway known as the PCP pathway. The PCP pathway consists of global directional cues, the cellular components that receive and interpret the global cues, and the effectors that execute the morphological polarization for individual cells. Directional information from global guidance cues is interpreted by the cell to establish the planar polarity axis with the help of downstream effectors coordinating machinery for cytoskeletal reorganization. Upon global cue guidance, the first step in achieving the coordinated orientation in cells is the asymmetric sorting of receptors and associated proteins, known as core PCP proteins,

across and along the planar surface of the cell. The polar localization of core PCP proteins interacts with effectors locally to carry out morphological changes. Hence, extracellular signals are transduced across the membranes by core PCP proteins and the signal is transferred to downstream effectors ultimately affecting cytoskeletal organization and structures.

Perturbations of the PCP genes result in a variety of defects over a wide range of organs. In the cochlea, PCP defects result in two major phenotypes: 1) convergent extension defects in which the cochlea is shorter with more than 4 hair cell rows intercalated with supporting cells and 2) loss of orientation of stereocilia bundles from the precise lateral pointed fashion typically seen within the cochlea (Kelly and Chen, 2007). Extraordinary examples of this phenomenon are present in core PCP protein mutants Looptail (Vangl2; Van Gogh Like 2), Crash and Spincycle (Celsr1; Cadhrein, EGF Lag seven-pass G type receptor 1) and knockouts of Fz3 (Frizzled3) and Fz6 (Frizzled6), Dvl (Disheveled), and a variety of PCP proteins that either directly or genetically interact with Vangl2 (discussed below) in which the cochlea becomes shorter and hair cells are oriented in a myriad of directions (Curtin et al., 2003; Kibar et al., 2001; Montcouquiol et al., 2003; Wang et al., 2005; Wang et al., 2006). Despite this pathway being involved in the establishment of polarity, studies using Vangl2 conditional knockout mice allowed researchers to determine that there is a Vangl2-independent orientation refinement processes occurring within the first 10 days after birth (Figure 1.2F) (Copley et al., 2013). Although this process is not completely understood, it's important to note that the system is often unable to correct gross misorientations with this postnatal refinement process.

PCP is directed through asymmetrically localized core PCP proteins including Celsr1, Vangl2, and Fz3 localized to the medial or lateral edges of the cell (Figure 1.3A-B) (Curtin et al., 2003; Montcouquiol et al., 2006; Wang et al., 2006). Celsr1 is localized to both the medial and lateral edges of cells and is required for the asymmetric localization of other core PCP proteins, likely playing a role in the trafficking of these proteins to the plasma membrane (Duncan et al., 2017). Vangl2, the most well studied of the PCP proteins due to the looptail mutant mouse, is localized to the lateral side of supporting cells according to stimulated emission depletion (STED) microscopy, opposite Frizzled localized to the medial edge of hair cells and supporting cells (Ezan and Montcouquiol, 2013; Montcouquiol et al., 2006; Wang et al., 2006). Studies have shown that Vangl2 mRNA is present in hair cells, but to date Vangl2 protein has not been definitely visualized in the hair cell, perhaps meaning that Vangl2 may also be localized to the lateral edge of the hair cell at some point during development (Montcouquiol et al., 2003). Based on a model from *Drosophila* studies, it is possible that Vangl2 and Fz3 interact extracellularly transducing a polarity signal between supporting cells and hair cells, supported by Vangl2 being needed for proper Frizzled localization to the medial hair cell membrane (Wang et al., 2006; Wu and Mlodzik, 2008). In addition to the PCP pathway, Fz plays a role in the canonical Wnt signaling pathway regulating transcription during development. While most Wnt proteins transduce their signal across the membrane through Fz and Dvl in the canonical pathway, Wnt5a has been found to be a ligand for mediolateral orientation of the cochlea suggesting these proteins may have selective roles in planar cell polarity signaling as well (Qian et al., 2007).

Core PCP proteins interact directly and indirectly with a variety of PCP associated protein and downstream effectors (Figure 1.3B). These downstream effectors help transduce cell-cell signals within the cell to confer polarity events in the cytoplasm. Initial research in *Drosophila* wing identified several core PCP proteins and their downstream effectors (Simons and Mlodzik, 2008). Subsequently, many of these downstream effector homologs have been found in mammals to help complete the PCP pathway in the mammalian systems. In *Drosophila* wing, core PCP proteins Vang (Van Gogh), Fz, and Fmi (Flamingo, homolog to Celsr1) were identified due to misorientation of bristles on the wing. PCP associated proteins and downstream effectors have been well established in this system. Fz interacts with disheveled (Dsh in *Drosophila*, Dvl in mammals) and Dgo (Diego), which stimulate actin polymerization to produce the wing bristle while Vang and Pk (Prickle) prevent actin polymerization through cytoskeletal regulating proteins (Simons and Mlodzik, 2008). In mammals disheveled (Dvl1/2/3) proteins are redundant proteins that pass PCP signals from Fz to regulators of actin and microtubule polymerization (Figure 1.3B) (Etheridge et al., 2008). Additionally, Ankyrin repeat domain 6 (Ankrd6) is a mammalian homolog of Diego (see Chapter 4) (Jones et al., 2014). Pk2 and Testin in mammals are homologs to Pk and are thought to compete for binding to Vangl2 with Dvl through negative feedback loops to regulate and inhibit actin polymerization (Figure 1.3B) (Simons and Mlodzik, 2008). These PCP associated proteins work directly and indirectly with downstream effectors to regulate actin and microtubule dynamics to coordinate the intrinsic polarity of *Drosophila* wing bristles and mouse sensory hair cells.

The Kinocilia and Intrinsic Polarity

Intrinsic polarity of a hair cell is marked by the positioning of the kinocilium and stereocilia in each individual hair cell. Intrinsic polarity within each hair cell is tightly coupled to the coordinated polarity of hair cells across the organ of Corti discussed above. The intrinsic polarity of each hair cell can remain intact upon disruption of PCP genes. The asymmetrical localization of core PCP proteins directs the coordinated orientation of the kinocilia and stereocilia as evident from PCP mutant mice including Vangl2-looptail mouse and Fz3/6 mutant mice that lack coordination between hair cells despite correctly formed stereociliary bundles (Montcouquiol et al., 2003; Montcouquiol et al., 2006; Wang et al., 2006). Despite of the lack of coordinated orientation of the hair cells in the cochlea in Vangl2 and Fz mutants, downstream effectors of PCP proteins and independent systems confer the intrinsic polarity of each hair cell allowing for the unique V-shaped stereocilia bundles with a single transient kinocilium at the vertex of each bundle.

The kinocilium is the nonmotile primary cilia of the sensory hair cell. The kinocilium emerges from the hair cell shortly after cellular differentiation of hair cells at E15.5 in mice (Figure 1.2B-C). The centrally located kinocilium migrates to the lateral edge of the hair cell. At the same time, stereocilia start to emerge from microvilli (Figure 1.2C) (Frolenkov et al., 2004). By E17.5 the kinocilium is aligned along the mediolateral axis as a read out of planar cell polarity with a neat V shaped stereociliary bundle with the kinocilia at the vertex (Figure 1.2D-E). Approximately ten days after birth (postnatal day 10- P10), the kinocilium retracts (Figure 1.2F).

Recently, several proteins regulating microtubules and the cilia have been identified that help elucidate the process of ciliation in hair cells. Lis1 (Lissencephaly-1), a microtubule regulating protein regulating dynein plus end motors on microtubules mediates the localization of the centrosome in the hair cell. This localization is mediated downstream of the PCP proteins through activation of Rac-PAK signaling allowing polarization of the basal body to the lateral edge of the hair cell thus affecting the planar polarity of the cell (Sipe et al., 2013). Likewise Kif3a, a kinesin microtubule motor protein, is vital for kinocilia development, but regulates the apicobasal localization of the centrosomes within the cell (Sipe and Lu, 2011). Additionally, Lis1 and Kif3a mutants have classic PCP phenotypes including mislocalized kinocilia, orientation defects, and, additionally, flattened stereocilia, suggesting a loss of signal between PCP proteins and kinocilia and intrinsic polarity machinery (Sipe et al., 2013; Sipe and Lu, 2011).

Intraflagellar transport protein 88 (IFT88) is a component of cilia trafficking particles allowing for cargo movement within the cilia, vital for kinocilia formation (Jones et al., 2008). IFT88 knockout cochlea displays PCP defects and has altered intrinsic polarity with the stereocilia losing its pointed polarized V shape turning into a flattened stereocilia or a circular bundle with no intrinsic polarity (Jones et al., 2008). Additionally, core PCP protein localization is retained in IFT88 mutants, placing the intrinsic polarity determinants conclusively downstream of core PCP proteins (Jones et al., 2008). Bardet-Biedl Syndrome (BBS) proteins involved in the developing cilia in many organ systems were also found to regulate the coordination of cells (PCP) and the formation of intrinsic polarity of hair cells (Ross et al., 2005).

It has been clearly demonstrated through PCP mutants such as looptail mice that the localization of the kinocilia in hair cells is a readout of PCP. The studies of the cilia in IFT88 knockout and BBS mutants indicate that the kinocilium itself is responsible for the intrinsic polarity within the hair cell. Subsequently, it has been determined that the kinocilium regulates the intrinsic polarity of the stereocilia bundle through the localization of apical proteins that seem to demarcate the boundary of different regions of the hair cell ultimately resulting in creating the blueprint for the contour of the stereociliary bundle (Bhonker et al., 2016; Tarchini et al., 2013). Thus, the kinocilium links coordinated polarity with the intrinsic polarity of the stereocilia bundle in hair cells.

Additional Intrinsic Polarity Proteins

In order to regulate intrinsic polarity, a group of proteins classically responsible for spindle organization come together on the apical surface to demark the areas medial and lateral to the stereocilia bundle before the stereocilia have matured. These molecular blueprint proteins include LGN (for its leucine-guanine-arginine repeats), the mammalian homolog to Pins; mInsc, mammalian Inscuteable; and $G\alpha_i$. These proteins demark what will become the “bare zone” of the sensory hair cell lateral to the stereocilia (Tarchini et al., 2013).

During hair cell morphogenesis, the hair cell is covered in microvilli except for the area directly next to the kinocilia (Figure 1.2B). Expression and localization of LGN and $G\alpha_{i3}$ to the lateral edge of the hair cell precedes migration of the kinocilium to the middle of the cell (Figure 1.2C) (Ezan et al., 2013). Alternatively, when the kinocilium is ablated with a cilia mutant, $G\alpha_i$ expression is expanded medially suggesting that kinocilia helps direct the positioning of these blueprint proteins (Bhonker et al., 2016;

Ezan et al., 2013; May-Simera et al., 2015; Siletti et al., 2017). Stereocilia bundles form from microvilli with the kinocilium at the vertex while the space lateral to the stereocilia bundles undergoes a regression of microvilli leaving the area bare (Figure 1.2D). The molecular blueprint proteins are thought to regulate the formation of these structures (Figure 1.3C). Specifically, mInsc is necessary for proper extension of the bare zone, LGN determines the V-shaped hair bundles, and $G\alpha_i$ may help to bridge PCP signaling to stereocilia patterning (Tarchini et al., 2013). Additionally these bare zone proteins restrict localization of aPKC to the opposite side of the cell, medial to the stereocilia (Figure 1.3D) (Tarchini et al., 2013). Notably, aPKC enrichment is thought to recruit and activate Ezrin (homolog to Radixin found in the cochlea) in enterocytes leading to the formation of microvilli suggesting aPKC may be activating the elongation of microvilli to form stereocilia (Saotome et al., 2004; Wald et al., 2008). LGN and $G\alpha_i$ also seem to play a role in stereocilia elongation as they have been identified in the tips of the longest stereocilia row in the developing hair cells and have been implicated in the elongation of stereocilia in development (Tarchini et al., 2016). In these ways, the classic spindle orientation proteins are directing the intrinsic polarity of sensory hair cells.

While the PCP pathway is linked to the intrinsic polarity molecular blueprint proteins, other factors also help link these pathways to coordinate the intrinsic polarity between each cell. Daple (disheveled associated protein with a high frequency of leucine residues) interacts with both the PCP protein Dvl at the lateral membrane and intrinsic polarity protein $G\alpha_i$ allowing these two pathways to work together to acquire the correctly oriented V-shaped bundle (Siletti et al., 2017). However, it appears the actual contour of the stereocilia bundle is regulated by the localization of the kinocilia in

relation to the $G\alpha_i$ marked area of the cell. When the kinocilium is located in the middle of the $G\alpha_i$ area of the cell, the correct contour forms, but, if the kinocilium is located to the side or center of the area, a deformed stereocilia bundle results (Siletti et al., 2017).

Building the Stereocilia

Once the signals have been received to build stereocilia from microvilli, downstream effectors must regulate actin polymerization to build the 20-300 actin filled stereocilia (El-Amraoui and Petit, 2005). In order to achieve this, the loosely packed microvilli elongate and widen to form densely packed hexagonal structures consisting of up to 3000 actin filaments (DeRosier et al., 1980; Tilney et al., 1980). Elongation is achieved by adding and organizing β-actin and γ-actin throughout the core of each stereocilium (Bretscher and Weber, 1978; Frolenkov et al., 2004). The elongated stereocilia taper at the cell body to form rootlets that insert into the cuticular plate, a gel like actin network allowing for the anchoring of the stereocilia (Furness et al., 2008). Actin bundlers, actin crosslinkers, actin nucleators, and motor proteins are all needed to regulate the formation and maintenance of stereocilia. Particularly, the protein Espin acts as a crosslinker of actin filaments to stiffen and elongate stereocilia as seen by its mutation in the Jerker mouse causing short stereocilia that quickly degenerate (Sjostrom and Anniko, 1992; Zheng et al., 2014; Zheng et al., 2000b). Fimbrin, Plastin, Whirlin, Diaphanous and molecular motors MyosinVI, MyosinXV, and MyosinVIIa are also needed for elongation of stereocilia (Belyantseva et al., 2003; Frolenkov et al., 2004; Higashida et al., 2004; Mburu et al., 2003). The ERM family of proteins including Ezrin expressed in early hair cell development, Radixin expressed in hair cells through life, and Moesin not expressed in hair cells crosslink the actin filaments of the stereocilia

to the plasma membrane of each stereocilia. Defects in Radixin cause fragmentation of the stereocilia bundle in hair cells (Kitajiri et al., 2004). Actin regulating proteins such as Rho family GPTase Rac1 and Cdc42 also are needed for the regulation of actin dynamics in the hair cell. When lost, mis-shapened cells, convergent extension defects, PCP defects, stereocilia bundle fragmentation, and absence or alteration of the kinocilia location are seen (Grimsley-Myers et al., 2009; Kirjavainen et al., 2015). This implicates general actin regulating proteins as vitally important not only in stereocilia formation, but in many aspects of hair cell development.

Stereocilia Adhesion Complexes and Usher Proteins

In addition to proteins that regulate elongation and bundling of actin in the stereocilia, adhesion proteins, scaffold proteins, and motor proteins are vital for keeping stereocilia attached to each other and build the mechanotransduction apparatus for the hair cells. The machinery that builds the mechanotransduction apparatus within the stereocilia was revealed from studies of Usher syndrome. Usher syndrome is a genetically diverse disease with mutations in at least 10 proteins from 12 loci resulting in sensorineural hearing loss and pigmentary retinopathy affecting 50% of the deaf-blind population (Keats and Corey, 1999). Mutations in the Usher complex proteins cause dysmorphic stereocilia including bundles that are fragmented, altered in length and width and may have malformed kinocilia ultimately perturbing development and maintenance of the mechanotransduction apparatuses of the sensory hair cells. Additionally, Usher proteins are localized to the synapse of hair cells and may play a role in synaptogenesis (Keats and Corey, 1999).

Through decades of research it has been concluded that the defects causing deafness in Usher syndrome are due to the deformation of stereocilia and, in particular, the loss of adhesive links between stereocilia. These adhesive molecules may be sanctioning signaling events possibly affecting the morphogenesis of the cytoskeleton as well as mechanotransduction within the stereocilia. Early in stereociliary bundle development, different Usher proteins localize to the tip of the stereocilia to build connections between individual stereocilia and between the stereocilia and kinocilia. Particularly, homodimers of Cdh23 (Cadherin23) and Pcdh15 (Protocadherin15) adhere extracellularly to form tip links connecting the tips of stereocilia between rows, kinociliary links linking the kinocilia to the adjacent stereocilia, and transient lateral links forming horizontal links between individual stereocilia (Goodyear et al., 2005; Michel et al., 2005). In addition to Cdh23 and Pcdh15, lateral links in the OHC rows contain Stereocilin (Verpy et al., 2011). These links first appear at E17.5 while stereocilia are still forming. While tip links remain present through the life of the hair cell, lateral links and kinocilia links are lost within the first week after birth. Concurrent with the loss of lateral links at P2, ankle links at the base of the stereocilia composed of VLGR1 (Very large G protein coupled receptor-1), Ushrin, Vezatin, and Whirlin are present but are lost by P12 (Cosgrove and Zallocchi, 2014; Goodyear et al., 2005; McGee et al., 2006; Michalski et al., 2007). Horizontal top connectors form between P9 and P12 and remain present through life (Goodyear et al., 2005). Thus, in adulthood, only the tip links and horizontal top connectors remain. These linkages not only allow for the cohesion and stiffness of individual stereocilia to one another to prevent fragmentation, but tip links are also responsible for mechanotransduction (Hackney and Furness, 2013).

Tip links connect the uppermost region of the stereocilia of the tallest stereocilia row to the uppermost region of the stereocilia in the next tallest row. At the location of insertion of the tip links are two densities in the stereocilia termed the upper tip link and lower tip link. The upper tip density located in the taller of the stereocilia contains transmembrane protein Cdh23 interacting with scaffold proteins Harmonin-b and SANS (Scaffold protein containing ankyrin repeats and SAM domain) (Adato et al., 2005; Grillet et al., 2009; Reiners et al., 2005). Harmonin couples the tip links to actin with its actin bundling role. At the lower tip link density located at the shorter stereocilia of the linkages, Pcdh15 interacts with proteins such as TMC1, TMC2, TMHS, and TMIE possibly composing or interacting with the mechanoelectrical transduction channel which has yet to be identified (Longo-Guess et al., 2005; Maeda et al., 2014; Zhao et al., 2014). Together, these proteins develop properly formed cohesive stereocilia and allow mechanotransduction.

Hearing and Auditory Perception

The organ of Corti is responsible for transforming sound waves and vibrations received by the sensory hair cells to electrical signals relayed through the auditory neural circuit to the brain cortex for sound perception. In humans, auditory perception is established in utero while in the mice onset of hearing occurs between P10 and P12 while continuing to develop for the first three weeks posnatally (Ehret, 1977). To achieve audition, sound waves from the environment are funneled into the auditory canal, transfer their vibrations to the middle ear bones via the eardrum or tympanic membrane. The middle ear bone, the stapes, then transfers the vibrations by pushing the oval window creating a pressure wave into the perilymphatic fluid of the scala

vestibuli. This wave travels through the scala vestibuli and then scala tympani. The flexible basilar membrane containing the organ of Corti moves with the pressure wave when traveling through the scala tympani (Figure 1.1B) (Hudspeth, 2000a). Movement of the organ of Corti upon the basilar membrane causes stereocilia within the hair cells to move through the endolymph of the scala media or cochlear duct causing a deflection of stereocilia in the IHCs against fluid and a deflection of the OHCs against the tectorial membrane (Figure 1.1B). Deflection in the direction of the tallest stereocilia pulls upon the tip links opening a non-selective ion channel where K^+ and Ca^+ ions move from the endolymph into the cell (Hudspeth, 2000b). This creates a depolarization event producing a potential that is moved from the tip of the stereocilia through the body of the hair cell to the cell base where the cell released excitatory neurotransmitters to nerve fibers (Hudspeth, 2000b). The deflection in the direction of the shorter stereocilia is thought to cause the mechanotransduction channel to close reducing the flow of ions and causing a hyperpolarization event leading to a loss of excitatory signals to the adjoining neurons. The signals given off from the sensory hair cells of the organ of Corti travel through the 8th cranial nerve to the brain for processing and decoding of the electrical signals resulting in the perception of sound (Hudspeth, 2000a). This process of auditory perception is incredibly sensitive and allows for discrimination of a 0.2% difference in sound frequencies and allows hearing of incredibly soft sounds when at its best (Dallos, 1996). In order for this to occur however, the organ of Corti and upstream and downstream components of audition must form properly and maintain their precise structures.

Summary

Development of the sensory hair cells in the organ of Corti relies on signaling events from embryonic development and through adulthood to establish and maintain their intrinsic and coordinated polarity. Each hair cell has precise apical structures including a V-shaped stereocilia bundle critical for the transmission of sound from vibrations to an electrical signal passed to the brain. The development of the sensory hair cell has been studied for decades with the past few decades' research focusing on the molecular pathways that shape and orient the hair cell's apical structures. Through these studies, the PCP pathway originally identified in *Drosophila*, has been found to establish the coordinated polarity between cells. Intrinsic polarity on the other hand is established through compartments of proteins on the apical surface creating a "molecular blueprint" for the formation of apical structures. Likely mediated by the kinocilium and cytoskeletal regulators, the "molecular blueprint" of the hair cell mediates stereocilia formation. The stereocilia are held together by adhesion complexes that also transmit deflection caused by sound vibrations within the cochlea leading to mechanotransduction and auditory perception.

Although these pathways involved in cochlear development are actively being studied, much remains to be explained. Particularly, the question of how the proteins of these pathways are selectively transported to their final destinations is not understood. Apical-basal and planar polarized proteins must be selectively transported to their correct domains. The next chapter of this dissertation discusses cellular trafficking in the context of the organ of Corti particularly the Rab11 family of proteins to help build a context for the research discussed in Chapters 3-5.

Figures

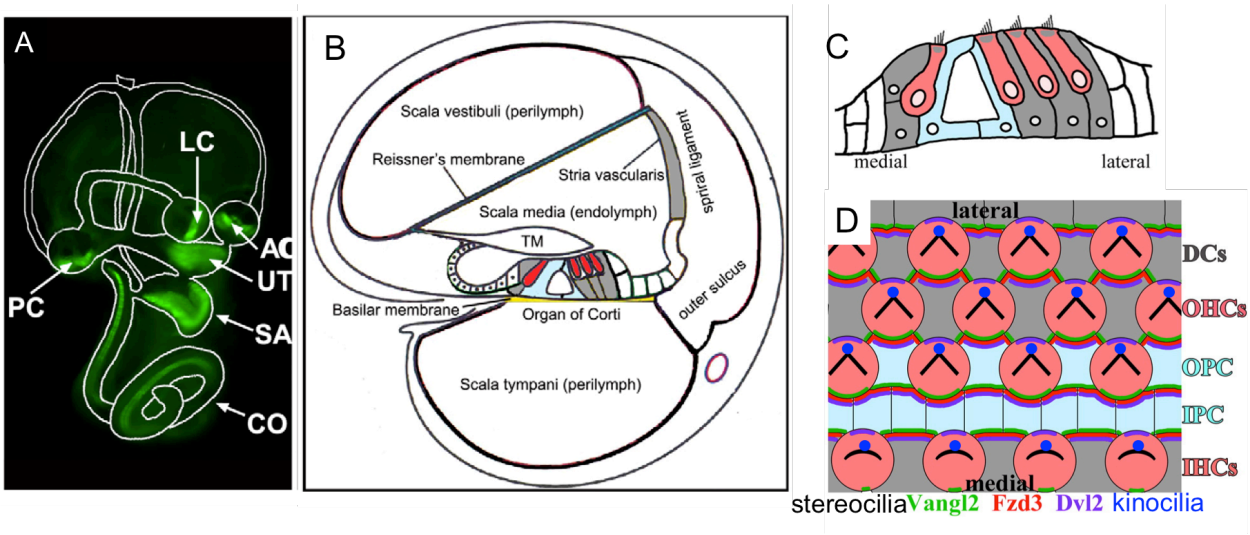

Figure 1.1 The inner ear and the Organ of Corti in the Cochlea

(A) The Inner Ear. The inner ear is composed of the cochlea (CO) and vestibular organs the saccule (SA), utricle (UT), posterior cristae (PC), lateral cristae (LC) and anterior cristae (AC). The sensory organs are marked by green fluorescent protein (green) with the labyrinths outlined.

(B) Anatomy of the Cochlea (cross sectional diagram). The cochlea a bony labyrinth containing membranous fluid filled labyrinths including the scala vestuli, scala tympani and the scala media (cochlear duct). The basilar membrane separates the scala tympani from the scala media. Lying on top of the basilar membrane is the organ of Corti. TM: Tectorial Membrane.

(C) The Organ of Corti (cross sectional diagram). The organ of Corti is composed of sensory hair cells (pink) alternated with supporting cells (light blue, grey). The three rows of outer hair cells (lateral) and single row of inner hair cells (medial) are separated by inner and outer pillar cells (light blue). At the apical surface of inner and outer hair

cells are graded rows of adapted microvilli, stereocilia, with the lateral most row being the tallest.

(C) The Organ of Corti (whole mount diagram). Sensory hair cells (pink) are interspersed in the organ of Corti with supporting cells (DCs- Dieter's cells, OPC- outer pillar cells, IPC- inner pillar cells). Both hair cells and supporting cells contain asymmetrically localized planar cell polarity proteins including Vangl2, Fz3, and Dvl2. Hair cells contain a V-shaped stereocilia bundle pointing laterally with a true primary cilia, termed the kinocilium, at the vertex. The three rows of outer hair cell (OHC) rows have a distinct V-shape while the inner hair cell (IHC) row has more of A U shaped stereocilia bundle.

Modified from Kelly, Chen, 2009.

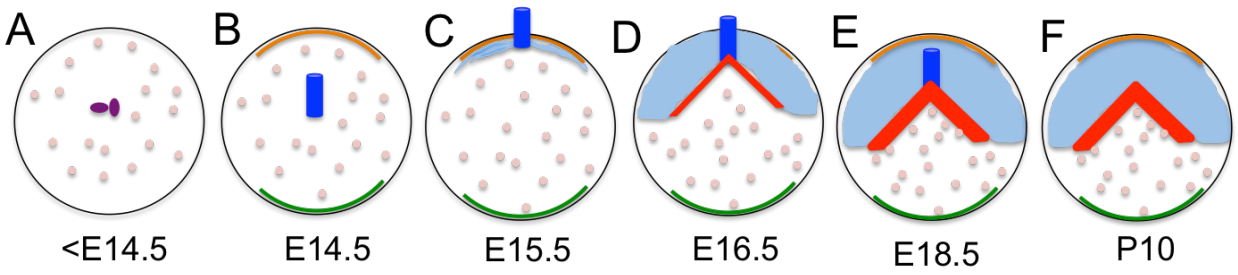

Figure 1.2. Development of the polar hair bundles in the Organ of Corti

(A) Post mitotic hair cells contain microvilli (pink). The basal bodies (purple) positioning determines the location where the kinociliium will form.

(B) By embryonic day 14.5 (E14.5) in mice, planar cell polarity proteins asymmetrically localize to the medial (green) and lateral (orange) edges of the apical membrane of the cell and the kinocilium emergences in the center of the cell.

(C) The kinociliium migrates to the lateral edge and the "bare zone proteins," LGN, $G\alpha_{i,}$ and mInsc (light blue) begin to express at the lateral edge of the hair cell

(D) By E16.5 the kinocilium starts to retreat back to center of the cell. Stereocilia (red) start to form the microvilli processes. The bare zone proteins mark the area lateral to the stereocilia where microvilli retract to form a microvilli free zone.

(E) By E18.5 the kinocilium moves further to the center of the hair cell and the stereocilia continue to form and refine to form the stereocilia bundle.

(F) During the first week postnatal the hair cell undergoes orientation refinement independent of Vangl2 and the kinocilium retracts by postnatal day 10 (P10).

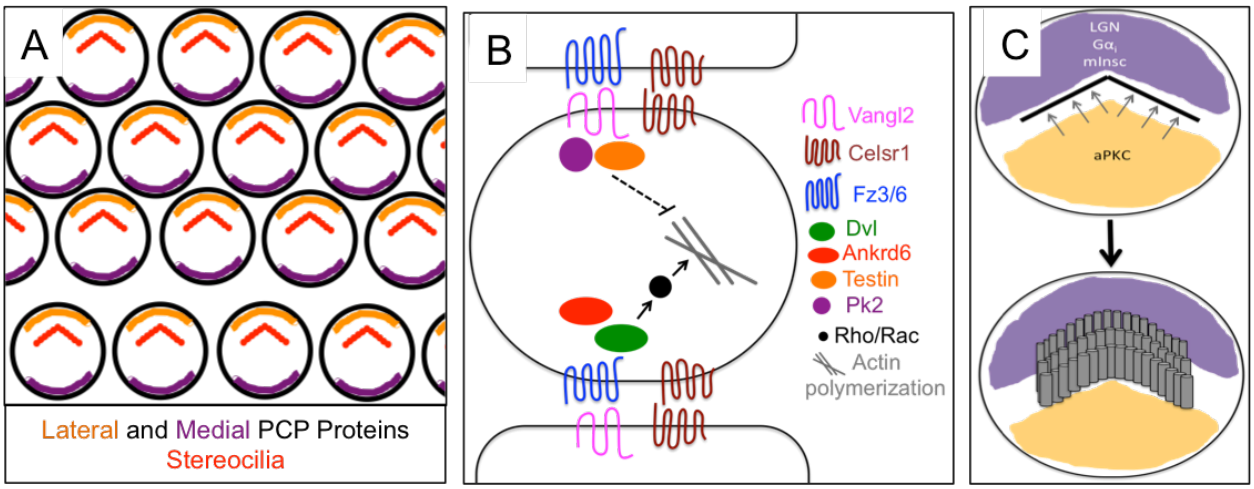

Figure 1.3. Planar Cell Polarity (PCP) in the Organ of Corti Hair Cells

(A) Coordinated Polarity of the Hair Cell is Directed by Asymmetrically Localized PCP Proteins. Asymmetrically localized PCP proteins differentially localize to the medial or lateral edges of the sensory hair cells and supporting cells (not pictured). The signal is thought to propagate between cells through extracellular interactions of PCP proteins. The PCP pathway influences the intrinsic polarity or orientation of the stereocilia bundle within each hair cell.

(B) A Model for PCP in the Hair Cell. Core PCP protein Celsr1 is expressed to both the medial and lateral edges of the hair cell (depicted as circular) allowing for the recruitment of Vangl2 and Fz3/6 to the cellular membrane. Extracellular interactions between Vangl2 and Fz3 propagate the PCP signal between cells. Despite only being able to visualize Vangl2 in the supporting cells (depicted as square) it may be localized to the hair cell as well. Dvl1/2/3 and Ankrd6 interact with Fz3/6 to localize to the medial side of the hair cell. Dvl activates Rho and Rac GTPases that regulate actin polymerization in the cell possibly influencing the building of stereocilia. At the lateral surface, Testin and Pk2 interact with Vangl2 and are thought to inhibit actin

polymerization. Additionally, Testin and Pk2 are thought to complete with Dvl for binding to Vangl2 to restrict Dvl to the medial membrane.

(C) PCP and Stereocilia Building Directed by Compartmentalization Proteins. A series of proteins termed “Molecular Blueprint” proteins classically implicated in spindle orientation segregate into discrete compartments on the apical surface of the hair cell. LGN, mammalian Inscrutable (mInsc), and $G\alpha_i$ are expressed at the lateral apical surface of the hair cell prior to stereocilia formation and mark what will be the bare zone. These proteins shape the hair bundle contour and restrict Atypical Protein Kinase C (aPKC) to the medial apical surface. aPKC recruits ERM proteins including Radixin and Ezrin necessary for microvilli formation leading to elongation of the stereocilia in the hair cell.

CHAPTER 2: Rab11a And Trafficking In Cell Biology

As outlined in Chapter 1, the cochlea's organ of Corti is composed of sensory hair cells interdigitated with supporting cells. The protrusions, stereocilia and kinocilia, that arise on the apical surface of the hair cells are vital for development and the mechanotransduction properties of the sensory hair cells. The precisely regulated delivery of proteins to the apical surface of hair cells is required for the formation of the kinocilia and stereocilia. In addition, specific and polar targeting of PCP proteins in the apical domain across the planar polarity axis is necessary to establish the uniform orientation of hair cells in the organ of Corti, a critical feature to support the functional sensitivity and capacity for audition. Although many aspects of the exact mechanisms of apical membrane trafficking remain elusive, several proteins have been identified in the apical trafficking pathways, most prominently members of the Rab11 family. Here I introduce Rab11, a protein involved in polar trafficking of proteins in epithelial cells.

In epithelial cells, apical-basal polarity is established through the highly regulated targeting and delivery of proteins to their final destinations. Proteins synthesized in the endoplasmic reticulum are modified in the Golgi and sorted for delivery in the trans Golgi network. At the trans Golgi network, coat-protein-mediated cargo sorting occurs to assure that proteins end in up at the correct cellular domain (Aridor and Traub, 2002). Differential transportation of apical and basal proteins ensues to allow for proteins to be delivered accurately to the correct cellular domain. A variety of small GTPases such as Rabs, Arfs, Arlfs, adaptor proteins, and motor proteins orchestrate the movement of cargos throughout the cell to facilitate cellular processes (Lim et al., 2011). The Rab11 family of proteins regulates the delivery of apical-basal polarized proteins. Cellular

extensions including cilia and microvilli sprout upon the apical surface of epitheilial cells. Through its role in trafficking, Rab11 regulates the formation and maintenance of these structures (Knodler et al., 2010; Knowles et al., 2015).

The Rab11 subfamily has three main members: Rab11a and Rab11b sharing a 91% identity differing only at the C-terminus and Rab25 (also called Rab11c) sharing 62 and 61 percent identity with Rab11a and Rab11b respectively (Welz et al., 2014). The similarities in these proteins have made distinguishing the differences in the roles of Rab11a and Rab11b difficult. Thus, much research into the function of Rab11 has failed to specify which Rab11 family member, particularly Rab11a and Rab11b, is implicated for many cellular events. Likewise, the roles of a particular Rab11 protein in cellular processes are often masked by the compensatory roles of its family members.

Rab11a in Protein Trafficking

The Rab11 family of proteins regulates and connects endocytosis and exocytosis through mediating the recycling endosome to allow proteins to be sorted and transported to distinct cellular membranes (Welz et al., 2014). Proteins endocytosed from the cellular membrane are sorted into vesicles differentially targeted to the lysosome for degradation or to the recycling endosome for redistribution to the cellular membrane (Maxfield and McGraw, 2004). If targeted for recycling, cargos are sorted into distinct vesicles for the common recycling endosome or the apical/pericentriolar recycling endosome (Perret et al., 2005). From there, the vesicles interact with the exocyst to specifically and precisely tether to the appropriate cellular membrane domain (He and Guo, 2009). Additionally, newly synthesized proteins from the trans Golgi network can be targeted to the recycling endosome for their delivery to the membrane

or exocytosis. Typically, Rab11 proteins involved in the apical recycling endosome delivering proteins to the apical membrane, but Rab11 has also been implicated in transcytosis of membrane proteins from one cellular domain to another and for the basolateral localization of Epithelial-cadherin (E-cadherin) (Lock and Stow, 2005). Although a lot remains to be elucidated, studies in cell culture have identified specific roles for Rab11 in protein trafficking.

Rab11 was initially described as the recycling endosome protein regulating membrane proteins such as transferrin in non-polarized cells and IgA in polarized cells to be endocytosed, sorted and redistributed to distinct domains of the plasma membrane (Casanova et al., 1999; Ducharme et al., 2007; Ullrich et al., 1996; Urbe et al., 1993). Likewise, Rab11 was found at the trans Golgi network directing newly synthesized proteins to the recycling endosome for delivery to the membrane such as targeting of proteins to the cilia (discussed below) and transport of vesicular stomatitis virus G to the basolateral membrane (Chen et al., 1998; Ullrich et al., 1996; Urbe et al., 1993; Yu et al., 2014).

Rab11 has been implicated in trafficking cadherins to the basolateral membrane. Originally, epithelial (E) Cadherin was found to rely on Rab11 for its basolateral localization, specifically from the Golgi through Rab11 positive recycling endosomes to the basolateral cellular membrane (Desclozeaux et al., 2008; Knowles et al., 2015; Lock and Stow, 2005). Similarly, *Drosophila* epithelial (DE) Cadherin relies on Rab11 for proper basolateral localization (Bogard et al., 2007). In germ line stem cells and embryonic ectoderm, Rab11 is required for cell polarity and tissue integrity by directing the localization of DE-Cadherin (Roeth et al., 2009; Xu et al., 2011). Localization of

neuronal (N) Cahderin also relies on Rab11 affecting neuronal migration (Kawauchi et al., 2010). Additionally, vascular endothelial (VE) Cadherin relies on Rab11a and the recycling endosomes for its localization through its interaction with Rab11 family interacting protein 2 (Rab11FIP2) forming a complex with Rab11a (Yan et al., 2016). While E, N, VE, and DE-Cadherins are all considered classical cadherins, they vary in their expression profile, function and, in the case of DE-Cahderin, their basic domain structure (Oda and Takeichi, 2011). Taken together, it appears that Rab11 directs the basolateral localization of a variety of Cadherins in multiple systems.

The movement of Rab11 positive vesicles relies heavily on motor proteins to transport the cargo across cytoskeletal tracks. Rab11FIP2 connects Rab11 to MyosinVb, an unconventional myosin motor protein allowing for Rab11 vesicles to transverse short distances across the dynamic actin cytoskeletal filaments (Casanova et al., 1999; Hales et al., 2002; Lapierre et al., 2001). In order to transverse long distances quickly, Rab11 interacting proteins Rab11-FIP3 and Protrudulin connect Rab11 vesicles to the microtubule minus end motor Dynein and the microtubule plus-end motor Kif5a respectively (Horgan et al., 2010; Matsuzaki et al., 2011). It is thought that Rab11 forms mutually exclusive interactions with Rab11 interacting proteins acting like scaffolds to help target the vesicles to the correct cellular domain (Jing and Prekeris, 2009). Once at the correct cellular domain, Rab11's interaction with exocyst complex component Sec15 allows for tethering and ultimately exocytosis or fusion of the vesicle to the correct membrane (Zhang et al., 2004).

Some studies have tried to elucidate the specific roles of Rab11a and Rab11b to demonstrate the separate functional roles each plays within the cell. For example, the

gastric H^+/K^+ APTase was found to localize to Rab11a positive vesicles but not Rab11b positive vesicles (Lapierre et al., 2003). Additionally, Rab11b localized to distinct apical pericentriolar regions compared to Rab11a and Rab11a's localization was affected to a greater extent than that of Rab11b when microtubules were disrupted (Lapierre et al., 2003). These data indicate that while the Rab11 family members may play partially redundant roles, each is responsible for distinct and specific cellular functions.

Rab11a in Cilia formation

Primary cilia are composed of a microtubule-based axonemes extending from the basal body surrounded by a specialized plasma membrane interspersed with receptors and signaling molecules (Lim et al., 2011). These receptors and signaling molecules as well as axoneme building machinery within the cilia must be selectively trafficked to and within the cilia. Interestingly, Rab11 recycling endosomes are present in high concentrations around the centrosome positioning it in the regulation of cilia transport (Perret et al., 2005). While the exact trafficking pathways of these proteins are unclear, we know that cilia targeted vesicles are sorted at the trans Golgi network for transport to the cilia and extensive progress into this trafficking event has been made in recent years (Figure 2.1) (Knodler et al., 2010; Wang et al., 2012). At the trans Golgi network, Arf4 sorts cilia bound cargo including Rabin8 into Rab11 positive vesicles (Knodler et al., 2010; Wang et al., 2012). These proteins form a cilia-targeting complex within vesicles with Rab11FIP3 and ASAP1 (Wang et al., 2012). Once at the basal body, Rab11 is able to activate Rabin8, which using its GEF activity, to activate Rab8 allows for membrane extension of the cilia and protein vesicle docking in conjunction with the BBSome, the Bardet-Biedel Syndrome protein complex (Jin et al., 2010; Knodler et al.,

2010; Nachury et al., 2007; Westlake et al., 2011). The BBSome in coordination with Arl6 is responsible for further sorting of cilia proteins and regulates entry into the cilia and transport to the cilia tip via interflagellar transport proteins leading to elongation and maintenance of the microtubule based cilia (Jin et al., 2010; Nachury et al., 2007). The coordinated trafficking of ciliary proteins, including Rabin8 to the cilia and subsequent entry and transport of proteins through the cilia is vital for the formation and maintence of the cilia in culture and *in vivo*.

Rab11a in Apical Surface Projections: Microvilli

Despite the progress that has been made in the Rab11 trafficking field, many papers have failed to elucidate the role of Rab11a vs Rab11b. Past studies have used knockdown of Rab11a/b or dominant negative constructs that may affect both Rab11a and Rab11b function. However, recent advancements in conditional knockout mice have allowed specific deletion of Rab11a (Knowles et al., 2015; Sobajima et al., 2014; Yu et al., 2014).

Recently studies in gut epithelia have revealed that Rab11a is vital for the apical protrusions of the intestinal epithelia, microvilli. With the loss of Rab11a in gut epithelia, microvilli atrophied leading to their reduction in length and width and a loss of apical specificity of microvilli (Feng et al., 2017; Knowles et al., 2015; Sobajima et al., 2014). These data clearly implicate Rab11a in the formation of apical microvilli structures. Specific knockout of Rab11a in gut epithelia explained Rab11a's role in the production of apical protrusions through its regulation of the apical distribution of several proteins including Ezrin; Mst4, an Ezrin phosphorylation protein; and STX3, an apical SNARE protein regulating apical membrane vesicular fusion; and MyosinVa, a motor protein

resulting in these proteins being irregularly targeted to both the apical and basolateral membranes (Knowles et al., 2015; Sobajima et al., 2014). Additionally, loss of Rab11a causes Rab11b and Rab8a to be dispersed throughout the cell body instead of localized to the apical cell domain and an upregulation of Rab11b (Knowles et al., 2015).

Trafficking and Rab11a's Potential Roles in the Inner Ear

Like many epithelial cells, the sensory epithelia composing the organ of Corti is highly polarized, particularly the sensory hair cells. The apical surface of sensory hair cells contains microtubule (cilia termed kinocilia) and actin projections (modified microvilli termed stereocilia) that rely on protein trafficking to be developed and maintained. Previous sections outlined the roles of Rab11 in building and maintaining these structures. In addition, the apical surface and membrane of the hair cell contains planar polarized domains that are vital for the proper development of the hair cell. Trafficking of PCP proteins, kinocilia proteins, stereocilia proteins, and signaling components of the apical membrane are vital for proper development of the hair cell leading to mechanotransduction. Although much of the trafficking activities in the cochlea remain a mystery, studies in the cochlea, other vertebrate systems, and cell culture have lead to advances that further implicate the importance of intracellular trafficking in hair cell development with some studies directly implicating Rab11 in these events.

Both inner and outer hair cells undergo endocytosis at the apical membrane allowing recycling of their apical membranes at least once every minute (Griesinger et al., 2002, 2004). Endocytosis allows for destruction of membrane components by the lysosome or recycling and redistribution of components via transcytosis and the

recycling endosome. In sensory hair cells, vesicles labeled with FM1-13, a marker of the cell membranes in IHCs and OHCs, are endocytosed at the apical membrane with IHCs trancytosing the vesicles to be released at the basal synaptic region of the hair cell and OHCs transcytosing the vesicles to the lateral domains of the cell membrane (Griesinger et al., 2002, 2004). In the IHC this activity of apical endocytosis increases during depolarization suggesting there is a link between synaptic activity and membrane recycling (Griesinger et al., 2002). In OHCs, the location of most of the endocytotic activity is located at the tallest stereocilia row near the basal body (Griesinger et al., 2004). Endocytosed vesicles enter early apical endosomes then move to common recycling endosome and apical recycling endosome for movement through the cell (Griesinger et al., 2004). This activity in OHCs may be involved in maintaining hair bundle integrity by recycling adhesion proteins in the stereocilia (Seiler and Nicolson, 1999). However, the specific trafficking pathways responsible for these endocytotic recycling are not well studied.

Interestingly, protein variants of adhesion proteins Pcdh15 and VLGR1 were found to undergo differential sorting to allow for apical and basal pools of each protein. The apical pool was found to be associated with the early endosome marker Rab5 while the basal pool was associated with AP1-positive-post trans Golgi vesicles and SNAP25 at the auditory synapse region of the cell (Zallocchi et al., 2012). It is thought that these two sub-pools separate functionally distinct variants of the adhesion proteins. Although the recycling endosome has been implicated in this process, the pathways downstream of the early endosome have yet to be elucidated.

Additionally, the gene ELMOD1 has been linked to deafness in mice and is a GAP for Arf6, a regulator of endosomal membrane trafficking, was identified in the cochlea (Johnson et al., 2012). By regulating Arf6, ELMOD1 is able to down-regulate apical endocytosis and membrane trafficking along with actin remodeling to stabilize stereocilia structures in the sensory hair cells of the cochlea and vestibular systems (Johnson et al., 2012; Krey et al., 2018). Specifically FM1-13 labeled membrane trafficking was disrupted in *rda* mutant mice with a ELMOD1 mutation leading to elongated and fused stereocilia that eventually degenerated (Krey et al., 2018). This positions endosomal trafficking upstream of stereocilia formation and its functional output, hearing.

The trafficking of PCP proteins responsible for coordinated orientation of sensory hair cells in the cochlea has also been studied *in vitro* and *in vivo*. Biosynthesized Vangl2 from the trans Golgi network is trafficked through interactions with Arfrp1 and AP1 (Guo et al., 2013). Although developed cells in the organ of Corti are post-mitotic, in dividing epidermis cells, PCP proteins are internalized and recycled. Celsr1 is recycled and recruits Vangl2 and Fz6 into Rab11a positive recycling endosomes and redistribution of the proteins following mitosis based on the neighboring cell's polarity, which could mean Rab11a is playing a similar role in post-mitotic cells (Devenport et al., 2011). Specifically in hair cells, Vangl2 is trafficked via endocytosis regulated by MyosinVI and Gipc1, a protein that regulates trafficking of transmembrane proteins and localizes to areas of "intense trafficking" although an exact mechanism has yet to be determined (Giese et al., 2012). Additionally, in *Xenopus* Rab11, regulates PCP during gastrulation, apical constriction neural tube closure and cell migrations (Kim et al., 2012;

Ossipova et al., 2015; Ossipova et al., 2014). Taken together, this suggests that Rab11 may play a role in PCP in through trafficking of Vangl2 and other PCP proteins or in other downstream aspects of PCP.

Summary

The organ of Corti is uniquely structured with apical projections vital for the proper development and function of the sensory structures, the stereocilia. Trafficking of the proteins needed for these structures must be vital for stereocilia formation, but the pathways of protein trafficking are largely unknown in the sensory hair cells. Rab11a is positioned to regulate kinocilia formation in the hair cell through delivery of Rabin8 to the cilia and subsequent activation of Rab8 at the cilia to allow for membrane extension (Knodler et al., 2010; Nachury et al., 2007; Westlake et al., 2011). Additionally, Rab11a has been implicated in the formation of microvilli in gut epithelia (Feng et al., 2017; Knowles et al., 2015; Sobajima et al., 2014). This coupled with known recycling of the IHC and OHC membranes particularly at the apical surface suggests Rab11a could play a role in stereocilia formation in the organ of Corti (Griesinger et al., 2002, 2004). Finally, experiments in non-mammalian systems have shown Rab11 to be involved in trafficking of PCP proteins and regulating PCP processes during embryonic development (Devenport et al., 2011; Giese et al., 2012; Kim et al., 2012; Ossipova et al., 2015; Ossipova et al., 2014).

Chapter 3 of this dissertation focuses on the role Rab11a plays in development of the organ of Corti by using Pax2-Cre driven knockout or Rab11a. Figure 2.2 discusses the possibly functions Rab11a could be playing in the organ of Corti hair

cells. Chapter 3 and 5 discuss how Rab11a affects hair cell development in the organ of Corti and proposes a model to explain the findings.

Figures

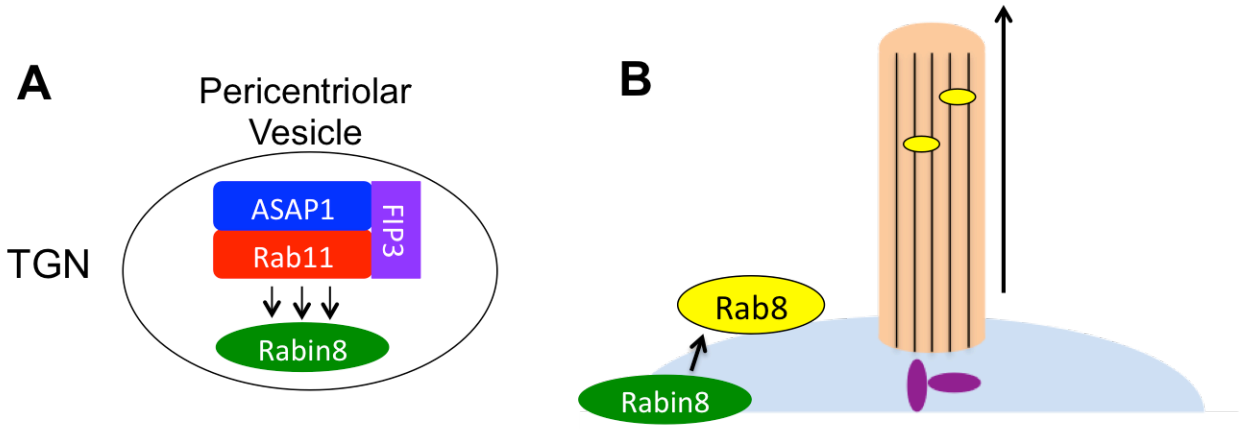

Figure 2.1 Rab11 in Ciliogenesis

(A) Proteins are sorted into vesicles at the trans Golgi network (TGN). Rabin8 is sorted into Rab11 positive vesicles where Rab11, ASAP1, and Rab11-FIP3 form a cilia-targeting complex. The pericentriolar vesicle containing these proteins are trafficked to the cilia. Subsequently, Rab11 activates Rabin8.

(B) Once at the cilia, actinvated Rabin8 uses its GEF activity to activate Rab8. IFT particles allow Rab8 to enter the cilia and extend the cilia membrane during axoneme elongation.

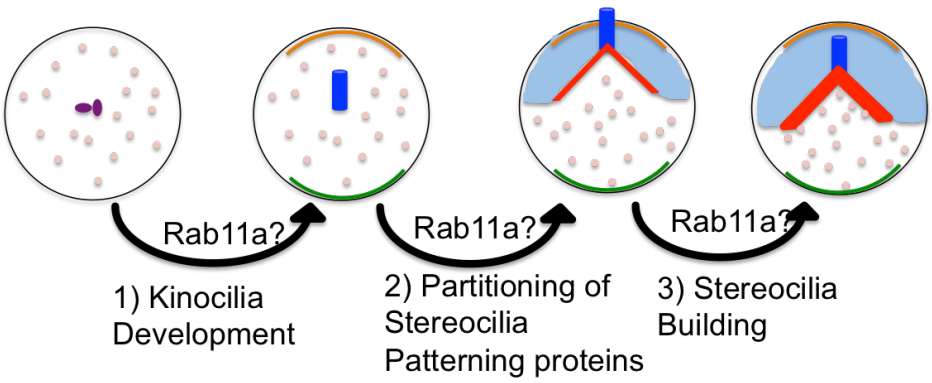

Figure 2.2. Model of Hypotheses of Rab11a's affect on the inner ear.

Rab11a could be playing several roles in hair cell development in the organ of Cori. First, Rab11 has been clearly implicated in cilia development *in vitro*. The organ of Corti hair cells rely on kinocilia formation for establishment of intrinsic polarity and stereocilia formation. Second, Rab11a could be affecting PCP protein distribution to their specific medial and lateral domains consistent with trafficking seen during recycling seen during mitotic internalization of PCP proteins. Third, Rab11a could affect stereocilia building similarly to what was observed in the gut epithelia in Rab11a conditional knockout animals.

CHAPTER 3: Rab11a plays a role in the development of the stereocilia and kinocilia in the mammalian organ of Corti.

This chapter is adapted from the following text in preparation for submission:

Rab11a plays a role in the development of the stereocilia and kinocilia in the mammalian organ of Corti.

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INTRODUCTION

The auditory sensory organ, the organ of Corti, housed within the cochlea on the flexible basilar membrane, is responsible for transforming sound waves and vibrations received by the sensory hair cells to electrical signals that are relayed through the auditory neural circuit to the brain cortex for sound perception (Dallos, 1996; Hudspeth, 2000a). The auditory system has high sensitivity that can discriminate a 0.2% difference in sound frequencies, and is capable of processing sound over a remarkably wide frequency and volume range in an instant (Dallos, 1996; Hudspeth, 2000a). The organ of Corti has a form of unique physical features of cellular arrangement and mechanotransduction apparatuses, to support the functional sensitivity, resolution, and adaptation of intensity and time of audition.

The organ of Corti is composed of four rows of sensory hair cells of two distinct types, the inner and outer hair cells. The inner hair cells (IHCs) are the primary cochlear transducers of sound having afferent innervation while the outer hair cells (OHCs) are endowed with motility, which is thought to allow for distinguishing frequency selectivity and innervate almost exclusively by efferent nerve fibers (Evans et al., 1991; Holley, 1996; Hudspeth, 1997; Ricci, 2003; Ricci et al., 2000; Wu et al., 1999). Consequently, the mechanotransduction apparatus on the apical surface of hair cells is precisely patterned for its function. Each hair cell is adorned with a hair bundle consisting of actin-filled stereocilia and a transit single microtubule based true cilium, the kinocilium. The stereocilia are graded in height, linked by inter-stereocilia and tip links, and patterned precisely into a V-shaped bundle with the tallest stereocilia at the vertex of the stereocilia bundle (Dallos, 1996; Hudspeth, 2000a). The kincilium, emanating from the

basal body is near the vertex of the stereocilia bundle, and is present transiently during development (Sobkowicz et al., 1995). All the hair cells within the cochlea coordinate their V-shaped stereocilia bundles to point to the periphery of the cochlear duct depicting the most distinctive form of planar cell polarity in vertebrates (Rida and Chen, 2009). This uniform orientation is vital as the directional deflection of the stereociliary bundle allows for the mechanoelectrical transducer (MET channel) located in the tips of the stereociliary bundles to open causing an action potential to be sent through the cell onto afferent neurons (Tompkins et al., 2017). The staircase arrangement, the cohesion between individual stereocilia, and the uniform orientation of stereocilia bundles provide a structural foundation for mechanotransduction with high sensitivity and resolution (Alagramam et al., 2001; Bolz et al., 2001; Johnson et al., 2003; Kitamura et al., 1992; Zheng et al., 2005).

The uniform orientation of hair cells in the cochlea is regulated by the planar cell polarity (PCP) pathway. PCP is directed through asymmetrically localized core PCP proteins including Vangl2 and Frizzled3 localized to the junction between the medial surface of hair cells and the lateral surface of supporting cells (Montcouquiol et al., 2006). The asymmetrical localization of PCP proteins directs the coordination of orientation of the kinocilia and stereocilia as evident from PCP mutant mice including Vangl2-looptail mouse and Frizzled3/6 mutant mice that lack coordination between hair cells despite correctly formed stereociliary bundles (Montcouquiol et al., 2003; Montcouquiol et al., 2006; Wang et al., 2006).

More recently, a group of proteins classically involved in spindle orientation has been identified as working with the PCP pathway to promote orientation and

development of the stereociliary bundles. This process termed the blueprint of hair cell outlines the contour of the stereocilia with mInsc/Lgn/Ga_i laterally demarking the bare zone and Par3/Par6/aPKC medially (Tarchini et al., 2013). This process appears to be downstream of PCP proteins but upstream of kinocilia positioning as Ga_i localization is dependent on PCP protein localization but Ga_i and LGN direct kinocilia migration during development (Bhonker et al., 2016; Ezan et al., 2013; Tarchini et al., 2013). In addition to the mInsc/LGN/Gai pathway for intrinsic polarity of each hair cell, the transient kinocilia functions with PCP genes to regulate the orientation of hair cells and stereocilia patterning (Jones et al., 2008). When the kinocilia is ablated, cells lose their coordinated polarity leaving stereocilia pointing in all directions, and lose intrinsic polarity with the stereocilia forming circular bundles that appear to have no planar polarity (Jones et al., 2008).

The machinery that builds the mechanotransduction apparatus, the hair bundles, was unveiled from studies of Usher syndrome. Usher syndrome is a genetically diverse disease where mutations in at least 10 proteins affect hearing, sight, and sometimes balance in patients (Cosgrove and Zallocchi, 2014). Mutations in the Usher complex proteins cause dysmorphic stereocilia including bundles that are fragmented, altered in length and width, malformed kinocilia ultimately perturbing development and maintenance of the mechanotransduction apparatuses of the sensory hair cells. Early in stereociliary bundle development, Usher proteins localize to the tip, base, or body of the stereocilia (Adato et al., 2005; Ahmed et al., 2003; Hasson et al., 1997; Holme and Steel, 2002; Johnson et al., 2003; Kitamura et al., 1992; Lagziel et al., 2005; Michel et al., 2005; Self et al., 1998; Washington et al., 2005). Additionally a variety of linkages

form between individual stereocilia and between the stereocilia and kinocilia. Particularly, Cadherin23 and Protocadherin15 herterodimerize to form tip links, kinociliary links, and transient lateral links between individual stereocilia (Cosgrove and Zallocchi, 2014; Kazmierczak et al., 2007). After birth the presence of ankle links composed of VLGR1 and Ushrin can be seen (Cosgrove and Zallocchi, 2014; McGee et al., 2006; Michalski et al., 2007). In adulthood, only the tip links and horizontal top connectors remain. These linkages allow for the development and adhesion of stereociliary bundles. Upon reaching maturity, the stereociliary bundles rely on the tip links for mechanotransduction. The upper tip density located in the taller of the stereocilia contains transmembrane protein Cadherin23 interacting with scaffold proteins Harmonin-b and SANS (Adato et al., 2005; Grillet et al., 2009; Reiners et al., 2005). Harmonin couples the tip links to actin with its actin bundling role. At the lower tip link density located at the shorter stereocilia of the linkages, Protocadherin15 interacts with proteins such as TMC1, TMC2, TMHS, and TMIE possibly composing or interacting with the MET channel (Longo-Guess et al., 2005; Maeda et al., 2014; Zhao et al., 2014). Together, these proteins develop properly formed stereocilia and allow mechanotransduction.

These studies have clearly demonstrated that sensory hair cells rely on interplay of signaling pathways and proteins in order to develop properly. While some genetic programs that govern the formation of key structural features of the mechanotransduction apparatus in the cochlea are known, it is not clear how proteins are specifically targeted to the unique locations within the hair cells or their stereocilia,

how the stereocilia building machinery interacts with the planar cell polarity complexes to erect the polar structure of hair bundles across the tissue.

In epithelial cells, the apical-basal polarity is established through the precise targeting and delivery of proteins to their final destinations. Proteins synthesized in the endoplasmic reticulum are modified in the Golgi and sorted for delivery in the trans-Golgi network. From there, apical and basal proteins are differentially transported to their respective domains. A variety of small GTPases such as Rabs, Arfs, Arlfs, adaptor proteins, and motor proteins orchestrate the movement of cargos throughout the cell to facilitate cellular processes (Lim et al., 2011).

The specification of projections, namely microvilli and cillia, to the apical membrane of epithelial cells is a common feature of the epithelial cells. The sensory hair cells of the organ of Corti may represent the most distinct and precise example in vertebrates. Proper sorting and targeting of the proteins to the apical membrane is vital for these projections to form. Although many aspects of the exact mechanisms of apical membrane trafficking remain elusive, several proteins have been identified in the apical trafficking pathways; most prominently, Rab11.

Studies *in vitro* have elucidated the mechanisms by which Rab11 positive vesicles are transport to the apical membrane (Welz et al., 2014). Rab11 delivers biosynthetic cargo to the apical surface through the trans Golgi network and redistribution of proteins through vesicles of the recycling endosome (Ullrich et al., 1996; Urbe et al., 1993). The movement of these Rab11 positive vesicles relies heavily on motor proteins to transport cargo across cytoskeletal tracks. Rab11-FIP2 (*Rab11 family interacting protein 2*) connects Rab11 to MyosinVb, an unconventional myosin

motor protein allowing for the transport of Rab11 vesicles (Casanova et al., 1999; Hales et al., 2002; Lapierre et al., 2001).

These transporting processes of cargo from the trans Golgi network and recycling endosome are crucial for the proper formation of cilia. Primary cilia are composed of a microtubule based axonemes extending from the basal body surrounded by a specialized plasma membrane interspersed with receptors and signaling molecules that must be trafficked to and within the cilia (Lim et al., 2011). While the exact pathway of ciliary proteins from the trans Golgi netowrk to the cilia is unclear, extensive progress has been made in understanding this process. Arf4 sorts cilia bound cargo at the trans Golgi network where the cargos, including Rabin8, are sorted into Rab11 positive vesicles (Wang et al., 2012). These two proteins form a targeting molecule with Rab11-FIP3 and ASAP1 (Wang et al., 2012). Once at the basal body, Rab11 is able to activate Rabin8, which, using its GEF activity, activates Rab8 allowing for membrane extension of the cilia and protein vesicle docking in conjuction with the BBSome, a protein complex responsible for regulating trafficking to the cilia named after Bardet-Biedl syndrome in which the associated genes are mutated (Jin et al., 2010; Knodler et al., 2010; Nachury et al., 2007; Westlake et al., 2011). The BBSome in coordination with Arl6 is responsible for further sorting of cilia proteins allowing entry into the cilia and transport to the cilia tip via interflagellar transport proteins (Jin et al., 2010).

Despite the progress that has been made in the Rab11 trafficking field, many papers have failed to elucidate the role of Rab11a vs. Rab11b. The Rab11 subfamily has three main members: Rab11a and Rab11b sharing a 91% identity differing only at the C-terminus and Rab25 sharing 62 and 61 percent identity respectively (Welz et al.,

2014). The similarities in these proteins have made distinguishing the differences in the roles of Rab11a and Rab11b difficult. Past studies have used non-specific knockdown of Rab11a/b or use of a Rab11a dominant negative thought to affect both Rab11a and Rab11b function. However, recent advancements in conditional knockout mice have allowed a Rab11a specific deletion (Knowles et al., 2015; Sobajima et al., 2014; Yu et al., 2014)

Specific knockout of Rab11a in gut epithelia has shown that Rab11a in fact does regulate the apical distribution of several proteins including Ezrin and Syntaxin3 and the formation of microvilli (Knowles et al., 2015; Sobajima et al., 2014). Additionally, Rab11a plays an essential role in guiding the secretion of matrix metalloproteinases in embryogenesis (Yu et al., 2014). However, it remains to be determined if these processes are consistent across tissue types. These studies have shown that Rab11a is necessary for development of the apical structures in the gut and play a role in specifying proteins to the apex opposed to the basolateral sections of the cell.

Considering these findings, we hypothesize that Rab11a regulates trafficking in the cochlea to affect the formation of the apical surface of the stereocilia and kinocilia in the organ of Corti hair cells. Using a Rab11a-floxed mouse in conjunction with Pax2-cre to drive recombination, we investigated the affect Rab11a on the development of cilia and stereocilia in the hair cells and cells in culture. Together, these methods allowed us to identify three roles of Rab11a in stereocilia formation: 1) formation of the kinocilia in conjunction with IFT88, 2) formation of stereocilia possibly through targeting of inter-stereocilia adhesion proteins, and 3) communication of planar cell polarity signals within the sensory hair cell.

Results

Rab11a is localized adjacent to the basal body in cochlear hair cells.

To start exploring the role of Rab11a in the cochlea, we investigated the localization of Rab11a in the cochlea. Stereocilia, actin rich modified microvilli were visualized with phalloidin while basal bodies, from which the kinocilia is derived, were stained using an antibody against γ-tubulin (Fig. 3.1A-E). Using a Rab11a-specific antibody, we found Rab11a localized near the basal body at the vertex of the V-shaped stereocilia bundles in sensory hair cells (HCs) (Fig. 3.1A-E). This localization is consistent with reports of Rab11 in the basal body in cultured cells and the cochlea (Kirjavainen et al., 2015; Knodler et al., 2010).

Both Rab11a and Rab11b mRNA were detected in cochlear epithelia throughout development (Fig. 3.1F). Due to the high identify in Rab11a and Rab11b proteins, we utilized an antibody reportedly raised against an epitope specific to Rab11a in its C-terminus (Cell Signaling Technology #2413). To confirm the specificity of the Rab11a antibody in the cochlea, we bred Pax2-Cre mice (Ohyama and Groves, 2004) to carry a floxed allele of Rab11a (Yu et al., 2014) to create Rab11a conditional knockout mice. We stained Rab11a conditional knockout (CKO) cochlea with the Rab11a antibody to determine antibody specificity. Rab11a signal was almost completely lost in Rab11a CKO cochlea confirming the specificity of the antibody and the efficiency of the Rab11a conditional knockout (Fig. 3.1G-J). Notably, a small fraction of inner and outer hair cells retain Rab11a staining consistent near complete conditional knockout of Pax2-Cre.

In summary, Rab11a was localized to the vicinity of the basal body in the cochlear HCs and its expression is mostly undetectable in Rab11a CKO mice.

Inactivation of Rab11a leads to defects in stereocilia bundles of HCs.

The significant reduction of Rab11a expression in the cochlear HCs of mice with Pax2-Cre driven inactivation of Rab11a allowed us this tool to analyze the role of Rab11a in the cochlea. We used these mice to investigate the cochlear phenotype in Rab11a CKO mice (Fig. 3.2). Each cochlear hair cell has precisely patterned hair bundles consisting of a staircase of stereocilia arranged in a V-shape and a kinocilia near the vertex of the V-shaped stereocilia. At the end of gestation (E18.5) control heterozygous littermates, hair bundles are recognizable in all hair cells (Fig. 3.2A). Strikingly, Rab11a CKO littermates at the same time point had missing stereocilia bundles and grossly deformed stereocilia bundles were observed in the IHCs and to a lesser extent in OHCs (Fig. 3.2B). At birth (P0) and P2 as the hair bundles achieve their distinct matured morphology, the stereocilia abnormality in the IHCs remains while a similar deformation of stereocilia bundles in the OHCs could also be more easily recognized although to a lesser degree (Fig. 3.2C-F).

We further quantified the stereocilia abnormality phenotype in both the inner (Fig. 3.2G-I) and outer hair cells (Fig. 3.3C), and classified the various abnormalities as missing bundles, circular bundle, flat bundle, and split or fragmented bundles (Fig. 3.2J, Fig. 3.3A). The stereocilia bundle abnormality manifests as missing or circular in 47.7% of the IHCs at E18.5, missing or circular in 23.6% and fragmented in 41.1% of the IHCs at P0, and fragmented in 66.9% of the IHCs at P2 (Fig. 3.2g-J, Fig. 3.3A). In conclusion, loss of Rab11a causes significant stereocilia bundle abnormalities in stereocilia bundles of the IHCs at E18.5, P0, and P2 (Fig. 3.2G-I).

The influence of Rab11a inactivation on hair bundle morphology varies along the longitudinal and mediolateral axes of the cochlea.

During cochlear development, HC differentiation and stereocilia bundle morphology maturation occur in a graded fashion from the base to the apex along the longitudinal axis of the cochlear duct and from inner to outer hair cells along the mediolateral axis of the cochlear duct (Montcouquiol and Kelley, 2003). To help us understand the stereocilia phenotype seen in Rab11a CKO cochlea, we analyzed the degree of stereocilia bundle morphology along the longitudinal and mediolateral axes. In control heterozygous cochlea, stereocilia bundles are V-shaped with nearly 100% normal stereocilia bundle morphology along the longitudinal axis (Fig. 3.3B). In Rab11a CKO cochlea at E18.5 there is very little difference in the percent of normal bundle morphology in the apex and base of the cochlea (Fig. 3.3B). However, as development continues postnataly and achieves more distinct morphology by P2, the percentage of normal hair bundles in mutants is markedly different from that of controls, and the difference is statistically significant (Fig. 3.3B). In addition, there is a difference observed in HCs along the mediolateral axis as well. In control IHC and OHCs there is no difference in the percentage of normal stereocilia as each genotype nears 100% normal stereocilia (Fig. 3.2G-I, Fig. 3.3C). However, there is a difference in IHC stereocilia morphology but not OHC stereocilia morphology between controls and Rab11a CKO cochlea (Fig. 3.2G-I, Fig. 3.3C).

Rab11a knockout in the cochlea produces a stereocilia bundle phenotype that is more prominent in the more mature cells in the base of the cochlear duct and inner hair cells compared to the less mature apex and outer hair cells.

Rab11a regulates ciliogenesis and interacts genetically with IFT88 in the formation of the kinocilia in HCs.

In culture and during *Xenopus* embryogenesis, Rab11 has been shown to regulate ciliation (Kim et al., 2012; Knodler et al., 2010). However, these studies do not distinguished differential roles for Rab11 family members. We sought to determine the function of Rab11a specifically utilizing the knockout technology of the Rab11a CKO mouse. First, we used mouse embryonic fibroblasts (MEFs) isolated from Rab11a-floxed mice in conjunction with a CMV promoter driven retrovirus to create Rab11a-null MEFs (Fig. 3.4A-E) (Yu et al., 2014). After inducing ciliation, MEFs without Cre activation (wildtype) express Rab11a at the basal body marked with γ-tubulin (Fig. 3.4A) and had well formed cilia marked with Arl13b (Fig. 3.4C). Rab11a-null MEFs had a loss of Rab11a staining consistent with the loss of Rab11a expression (Fig. 3.4B). Additionally, Rab11a-null MEFs have a significant loss of ciliation dropping from 97% ciliation in wildtype to 33% ciliation in Rab11a-null cochlea (Fig. 3.4D,E). Ciliation could be rescued by expressing wildtype Ds-Red-Rab11a suggesting that the result is specific to Rab11a (Fig. 3.4E).

The cochlea offers a unique opportunity to study the role of Rab11a in ciliogenesis *in vivo*. The kinocilia of the cochlea plays a vital role in stereocilia formation. When absent, stereocilia become circular similar to what we saw in Rab11a CKO hair cells (Fig. 3.2B,E) (Jones et al., 2008). Based on the observations of Rab11a's role in ciliogenesis *in vitro* and the observed phenotype of Rab11a CKO cochlea being similar to cilia mutant HCs, we hypothesized that Rab11a may play a role in kinocilia formation.

Sensory hair cells of the cochlea have a precisely oriented kinocilia at the vertex of each stereociliary bundle (Fig. 3.4F). In contrast with our *in vitro* results, Rab11a knockout in the cochlea did not ablate ciliation in IHCs or OHCs (Fig. 3.4G). Notably, we observed an uncoupling of the kinocilia from the vertex of the stereocilia in a fraction of IHCs (Fig. 3.4G). In the absence of a correctly formed stereocilia bundle, the kinocilia can be observed to the left or right of center instead of at the lateral edge (Fig. 3.4G, zoomed IHC).

Due to possibly functional redundancy with Rab11b or other ciliogenesis components, we used an established cilia mutant to functionally weaken the ciliogenesis pathway in the cochlea. IFT88 is a ciliation protein responsible for transport of cargo within the cilia. Ablation of this protein in the cochlea causes a severe loss of ciliation (Jones et al., 2008). To test the genetic interaction of Rab11a and IFT88 in the ciliogenesis pathway, we first used a double heterozygous conditional knockout of both Rab11a and IFT88 (Fig. 3.4H). The HCs in these cochlea appeared normal with kinocilia present in both inner and outer HCs. We then analyzed Rab11a homozygous CKO coupled with heterozygous CKO of IFT88 (Fig. 3.4I). These cochlea exhibited a significant decrease in ciliation in the IHC where approximately 50% of HCs lost their kinocilia (Fig. 3.4I,J). Similarly to the stereocilia phenotype, the OHCs were not significantly affected (Fig. 3.4I,K). Upon further analysis, ciliation did not present in a clear gradient; ciliation was apparently equal in the apex, middle, and base regions along the cochlear duct in both IHCs and OHCs (Fig. 3.5A,B).

Since the kinocilia has been established in stereocilia formation and patterning, we predicted that adding IFT88 heterozygous conditional knockout to Rab11a CKO

cochlea would cause an increase in the severity of the phenotype seen. Upon quantification of all IHCs along the cochlear duct, there was no clear difference between Rab11a CKO cochlea with or without IFT88 (Fig. 3.5C). However, when separated by region, we could see that the less severe phenotype in the apex was masking the marked differences in the base and middle regions of the cochlea with a 10% decrease in normal stereocilia in the base and a 12% decrease in the middle region (Fig. 3.5D). These data further support the role of kinocilia in stereocilia formation and connects Rab11a's role in the stereocilia and kinocilia.

Through the use of Rab11a conditional knockout technology, we were able to demonstrate that Rab11a specifically is necessary for ciliogenesis *in vitro*. In contrast, Rab11a is not necessary for kinocilia formation in the cochlea but may play a role in the orientation of the kinocilia. Further, Rab11a interacts genetically with IFT88 in kinocilia formation giving it a clear role in kinocilia formation. We also noted that compounding heterozygous IFT88 CKO to Rab11a CKO intensifies the stereocilia phenotype in the middle and base IHCs giving further evidence that the kinocilia regulates stereocilia formation.

Ultra structure of Rab11a reveals clear stereocilia fragmentation and kinocilia loss in the cochlea

In order to further characterize the phenotypes associated with loss of Rab11a in the cochlea, we turned to scanning electron microscopy (SEM). We analyzed cochlea isolated from P2 mice (Fig. 3.6A-C). Under SEM, wildtype cochlea have the characteristic V-shaped stereocilia bundle with the kinocilia at the vertex (Fig. 3.6A). Stereocilia generally couple together based on the lack of space between individual

stereocilia. However, SEM reveals that Rab11a CKO HCs experience fragmentation or a loss of adhesion between stereocilia with gaps or missing stereocilia between fragments of bundles in both IHCs and OHCs (Fig. 3.6B). Upon coupling Rab11a CKO with IFT88 heterozygous CKO, it is clear that there is a loss of cilia opposed to a reduction in kinociliary length in IHCs (Fig. 3.6C, IHC). We also note that there is no reduction in OHC kinocilia length. In these samples, stereocilia are largely fragmented and present with two sections of stereocilia: one at the lateral part of the cell, and another flatter bundle medially. Additionally, these HCs revealed the partial formation of a circular bundle in the OHC.

Taken together, these data suggest that Rab11a loss causes fragmentation of stereocilia possibly due to a loss of patterning of stereocilia adhesion. Additionally, kinocilia are not reduced in length in Rab11a CKO and are absent in Rab11a CKO coupled with IFT88 heterozygous CKO suggesting that there is a complete loss in kinociliation opposed to a reduction in kinocilia length.

Protein partitioning along the PCP and apical-basal axes of the organ of Corti is not apparently affected by Rab11a CKO.

Rab11 was first identified as a trafficking protein of the recycling endosome and trans Golgi network responsible for transport of proteins to the membrane (!!! INVALID CITATION !!! (Chen et al., 1998b; Ullrich et al., 1996)). Rab11a has been found to affect PCP processes such as gastrulation and neural tube closure in *Xenopus* during embryogenesis (Kim et al., 2012; Ossipova et al., 2015; Ossipova et al., 2014). Likewise, *in vitro* data suggest that Rab11 is involved in PCP protein trafficking (Devenport et al., 2011). Rab11 has even been implicated in trafficking Vangl2, a major

component of the PCP pathway (Kim et al., 2012; Ossipova et al., 2015; Ossipova et al., 2014).

First, we looked at localization of stereocilia patterning proteins in the PCP pathway. The PCP pathway directs the precise orientation of kinocilia and stereocilia orientation in HCs (Rida and Chen, 2009). PCP proteins such as Vangl2 and Frizzled3 are localized asymmetrically in hair cells and supporting cells along the mediolateral axis at the junction at the medial edge of hair cells and the lateral edge of supporting cells (Fig. 3.7C) (Montcouquiol et al., 2006). Similarly, in Rab11a CKO cochlea, retained the asymmetrical localization of Vangl2 and Frizzled3 along the mediolateral axis with no apparent differences from control cochlea (Fig. 3.7C,D). This data suggests that Rab11a is not necessary for the proper asymmetric mediolateral localization of Vangl2 and Frizzled3.

Secondly, we analyzed the localization of stereocilia patterning proteins that are thought to mark the localization of microvilli extension. The LGN and Ga_i proteins are localized lateral to the stereocilia in the hair cells in the bare zone where microvilli retract during stereocilia elongation (Tarchini et al., 2013). aPKC on the other hand is localized medially to the stereocilia in the area where microvilli remain after stereocilia elongation. Since the localization of these proteins precede stereocilia formation, they have been termed the “blueprint” for stereocilia patterning. Additionally, mutations or loss of function of these proteins perturbs stereocilia patterning. We analyzed LGN localization in wildtype and Rab11a CKO cochlea to determine if the stereocilia phenotype could be explained by mislocalization or loss of these stereocilia-patterning proteins (Fig. 3.7A-B). In control cochlea, LGN is localized to the region lateral to the

stereocilia (Fig. 3.7A). Surprisingly, LGN was expressed in the Rab11a CKO cochlea, even in IHCs that completely lacked stereocilia. This data suggests that the stereocilia defect we observe is downstream of the LGN/molecular blueprint-patterning pathway.

Rab11 has specifically been implicated in targeting of membrane proteins to the apex of the cell opposed to the basolateral surface. Since we did not see a difference in protein localization across the planar axis of the cochlea, we decided to look at the apical-basal cell axis of the cochlea. Using orthogonal views we were able to see the stereocilia, cuticular plate, and cell junction with phalloidin staining (Fig. 3.7A'-D'). LGN is localized to the lateral edge of each hair cell and is constricted to the apical most surface marked by the actin of the cuticular plate in both control and Rab11a CKO cochlea (Fig. 3.7A'-B'). The planar cell polarity proteins also localize to the apical half of hair cells in controls but on the medial edge in both gentoypes (Fig. 3.7C'D'). These data suggest that Rab11a is not affecting apical localization of stereocilia patterning proteins in the cochlear HCs.

In addition to molecular pathways being located on the apical surface of hair cells, there are apical structures to hair cells that are vital for their physiological function: hearing. Stereocilia are vital for the transmission of sound as they house the mechanoelectrical transduction channels triggered by the bending of the stereocilia. In order for the stereocilia to be formed properly, they must be anchored into the actin meshwork of the cell body, a structure termed the cuticular plate. Although we can see actin at the cuticular plate, we wanted to confirm that other major components were present. In both control and Rab11a CKO cochlear HCs, Spectrin is localized throughout the entire cuticular plate region with the exception of the fonticulous from

which the kinocilia emerge (Fig. 3.8AB). We also wanted to confirm that MyosinVIIa was present in the proper localization as it is a motor protein delivering cargo to the stereocilia during building and maintenance. Similarly, in both control and Rab11a CKO cochlear HCs MyosinVIIa is localized throughout the apical compartment of the cell with a large concentration around the cuticular plate and stereocilia as seen in the orthogonal view (Fig. 3.8BC). Finally, we looked at Radixin expression in the cochlea. Radixin links actin bundles in the stereocilia to the adjacent membrane and when ablated results in a similar phenotype where stereocilia bundles are fragmented (Kitajiri et al., 2004). Similarly to other proteins analyzed, control and Rab11a CKO HCs had similar localization of Radixin to the stereocilia of HCs and expression in supporting cells (Fig. 3.8EF). These data suggest that Rab11a is not solely responsible for global trafficking of proteins involved in actin organization of stereocilia.

Rab11a has also been found to be responsible for the basolateral localization of E-Cadherin (Desclozeaux et al., 2008). E-cadherin is expressed only in OHCs, while N-Cadherin is expressed embryonically in IHCs. In order to test if global Rab11a trafficking was altered we compared E-Cadherin expression in controls and Rab11a CKO cochlea (Fig. 3.9AB). In both, E-Cadherin is expressed at the cell membrane of OHCs (Fig. 3.9A-B). Looking at the orthogonal view, we saw clear basolateral expression with no apical expression in either sample (Fig. 3.9A'B') suggesting that Rab11a does not affect the ability to sort basolateral proteins in the hair cell.

In conclusion, planar polarity partitioning of Vangl2, Frizzled3, and LGN was not altered with loss of Rab11a. Likewise, little to no difference was seen in the apical localization of these proteins. Molecular components of the apical actin machinery of the

stereocilia including Spectrin, MyosinVIIa, and Radixin also retained apical localization with loss of Rab11a. Finally, E-Cadherin localization to the basolateral surface in OHCs was not altered. Taken together, these data suggest that loss of Rab11a does not cause a global loss of apical basal sorting ability despite clear role of Rab11 in trafficking in the literature.

Rab11a regulates PCP downstream of core PCP proteins.

Since the cochlea is a model for PCP with distinct orientation of stereocilia bundles, we sought to determine if Rab11a affects PCP in the cochlea. We observed that Rab11a does not alter the asymmetric localization of core PCP proteins (Fig. 3.7). In P0 wildtype cochlea hair cells, stereocilia are oriented with their V-shaped stereocilia bundles pointing laterally in a coordinated fashion (Fig. 3.2C). There was no apparent alteration in Rab11a CKO and control cochlea (data not shown). Because many PCP associated protein mutants do not show a severe PCP phenotype, we used a Vangl2-looptail mutant to determine if Rab11a is involved in any step of the PCP pathway. We weakening the PCP pathway with Vangl2-looptail heterozygous mutant and analyzed the apical section of P0 cochlea where hair cells are not as mature as the basal section. In heterozygous Vangl2-looptail controls, there is slight misorientation in the outermost hair cell (OHC3) (Fig. 3.10A,C,E). In the compound mutant however, there was more dramatic misorientation in the OHC row 2 and 3 (Fig. 3.10B,D,E). Quantification of the stereocilia angle revealed a significant increase in the proportion of misoriented hair cells (defined by 30˚ deviation or more from the mediolateral axis) in OHC3 and a slight increase in OHC2 rows in compound mutants compared to Vangl2-looptail controls (Fig. 3.10C,D). However, the IHC row and most medial OHC row had no difference in

misorientation. These data suggest that Rab11a may be playing a role in PCP signaling downstream of the core PCP proteins.

Rab11a is required for hearing and adults have hair cell degeneration

In order to determine the long-term effects of Rab11a loss of the cochlea, we analyzed 4 week old mice for hearing function and their cochlea to determine hair cell structure. Adult cochlea from the mice were dissected and stained with phalloidin. Wildtype mouse cochlea have OHCs with V-shaped stereocilia bundles and IHCs with U-shaped bundles and longer stereocilia (Fig. 3.11A). In Rab11a CKO cochlea, hair cell degeneration could be observed by the lack of stereocilia and a loss of MysoinVIIA expression suggesting hair cell degeneration and loss (Fig. 3.11B,C). Additionally, stereocilia appear more fragmented in both the IHC and OHC row with IHC stereocilia bundles also appearing severely less organized (Fig. 3.11B). Auditory Brainstem Response (ABR) threshold tests performed on these mice showed normal hearing range for control animals while thresholds were significantly higher for Rab11a CKO mice for all frequencies tested indicating profound hearing loss (Fig. 3.11C). These data indicate that Rab11a is responsible for the maintenance of both IHCs and OHCs and is necessary for proper hearing in adult animals.

Rab11a is required for Cadherin23 localization to stereocilia-like extensions in cultured cells

Further investigation of the fragmented phenotype of Rab11a CKO cochlea lead us to investigate the possibility that Usher protein localization was involved in phenotype seen by Rab11a CKO cochlea. Microvilli containing LLC-PK1-CL4 cells derived from pig kidney have been used in conjunction with transfection of Espin to induce elongation

of the microvilli that resemble stereocilia (Zheng et al., 2014; Zheng et al., 2010). We used this system to determine localization of Usher protein Cadherin23 in the presence of transfected WT and dominant negative Rab11a constructs (Fig. 3.12). In non-transfected and Rab11a-WT transfected cells, Cadherin23 was localized to the extended microvilli induced by Espin expression (Fig. 3.12A-B). In each instance only the apical section of the cell containing microvilli contained Cadherin23 (Fig. 3.12A'-B'). Cells transfected with Rab11a dominant negative construct, however, had Cadherin23 localized to the entire cell including the apical and basal regions of the cell (Fig. 3.12C,C'). This data is consistent with the hypothesis that Rab11a affects trafficking of Usher proteins in the hair cells.

Discussion

Until recently, progress in understanding the importance of Rab11a in cellular processes were achieved by using overexpression of dominant negative forms of Rab11a in cell culture (Casanova et al., 1999; Desclozeaux et al., 2008; Lock and Stow, 2005; Wilcke et al., 2000) or knockdown of Rab11a in culture or in vivo (Kim et al., 2012; Ossipova et al., 2015; Ossipova et al., 2014). While these methods have led to many discoveries of the Rab11 family, it is unknown whether the dominant negative constructs also force their dominance over Rab11b or other related proteins due to high amino acid sequence identity. Consequently, many papers report the role of Rab11 instead of Rab11a or Rab11b.

Recently two groups have created knockout mice where the Rab11a gene is floxed with loxp sites (Sobajima et al., 2014; Yu et al., 2014). Using one of these Rab11a knockout mice combined with Pax2-Cre to produce knockout in the inner ear,

we show that Rab11a, a protein primarily given the role of apical trafficking and ciliogenesis is vital for formation of the apical processes in the organ of Corti (Ohyama and Groves, 2004; Yu et al., 2014). Rab11a is localized to the basal body region of sensory hair cells and is vital for stereocilia patterning, particularly in IHCs. Additionally, Rab11a plays a clear but nonessential role in kinocilia formation as evident by a loss of ciliation when loss of Rab11a is coupled with deletion of one allele of *IFT88*. We demonstrated a genetic interaction between Rab11a and Vangl2 suggesting Rab11a participates in planar cell polarity downstream of core PCP protein localization. Rab11a loss leads to hair cell degeneration and hearing loss. To help explain this phenotype, we utilized a cell system to determine that Rab11a regulates apical trafficking of Usher proteins. Taken together, these results suggest that Rab11a is involved in several cellular processes more distinct than general apical-basal trafficking and ciliation.

Rab11a in apical protein localization

Rab11a was initially described as a protein of the recycling endosome and trans Golgi network responsible for apical protein trafficking in epithelial cells (Ullrich et al., 1996; Urbe et al., 1993). Additionally, Rab11a was found to be a major player in the recycling endosome allowing membrane proteins to be endocytosed, sorted, and redistributed to the plasma membrane allowing for trancytosis of proteins or a final distribution of proteins (Ducharme et al., 2007). This apical trafficking of proteins allows for cellular processes to take place and cellular structures such as actin and microtubule based projections to form precisely on the cell (discussed below).

In the organ of Corti the stereocilia form a distinct and uniform pattern on the apical surface of sensory hair cells. Utilizing antibodies against proteins involved in

patterning and formation of the stereocilia, we were unable to detect a change in apical localization of many proteins involved in the patterning of the apical surface including planar cell polarity proteins Vangl2, Frizzled3, and LGN and actin interacting proteins Spectrin, Myosin7a, and Radixin.

These results were surprising considering the phenotype of Rab11a CKO in the gut. Two separate alleles of Rab11a knockout in intestines resulted in mislocalization of apical proteins (Knowles et al., 2015; Sobajima et al., 2014). Specifically, Ezrin, the form of the ERM protein present in the gut was mislocalized laterally from the normal apical localization (Knowles et al., 2015). Radixin, the ERM protein expressed in the cochlea retained its apical localization in the actin rich cuticular plate and stereocilia. We also analyzed several apically expressed proteins vital for cochlear hair cell development but did not find mislocalization suggesting Rab11a is not solely responsible for apical trafficking in the cochlea.

Rab11a has also been implicated in the basolateral localization of E-Cadherin *in vitro* (Desclozeaux et al., 2008; Lock and Stow, 2005). Like the studies in the intestines and embryo, using Rab11a null mice, E-Cadherin retained basolateral localization in the cochlea upon the knockout of Rab11a (Feng et al., 2017; Knowles et al., 2015; Sobajima et al., 2014; Yu et al., 2014). These data suggest that Rab11a may play a role in E-Cadherin distribution in some systems, but in tissue, there may be redundancies that ensure E-Cadherin is localized appropriately for if it was not, cells would not adhere properly and development of the embryo and organs would be significantly altered.

Rab11a in ciliogenesis in the cochlea

The Rab11 family has been implicated in ciliogenesis through its interactions with Rabin8 that acts as a GEF for Rab8, necessary for membrane extension of the cilia (Knodler et al., 2010; Nachury et al., 2007). In order for ciliation to occur, cilia bound vesicles are sorted at the Golgi for transport to the cilia. Rab11 positive vesicles including Rabin8 destine for the cilia plasma membrane transports these vesicles (Knodler et al., 2010). These data point to a pivotal role for Rab11a in the localization of important ciliation factors to the cilia. However, these studies were done *in vitro* using nonspecific Rab11 knockdown. Here, using a Rab11a Knockout MEF line with complete loss of Rab11a specifically, we definitely show that Rab11a is necessary for ciliogenesis in MEF cell culture.

In vivo, Sobajima et al. (2014) determined that when Rab11a was knocked out in the brain using Nestin driven Cre recombinase, there was no effect on ciliation (Sobajima et al., 2014). Likewise, knockdown of Rab11a in the cochlea did not alter kinocilia formation and presence within the cell. When taken together with the vital role of ciliogenesis in development, it is not surprising that a loss of a single gene does not ablate ciliation. We chose to weaken the ciliation system in the cochlea using knockout of interflagellar transport protein, *IFT88*. When the ciliation pathway in the cochlea was weakened from heterozygous knockout, loss of Rab11a did cause nearly 50% loss of ciliation in inner hair cells of the organ of Corti. This suggests that Rab11a does play a vital role in cilation as IFT88 heterozygous knockout do not show a loss of ciliaiton. We hypothesize that the lack of a defect in ciliation in Rab11a knockout cochlea is due to redundancy or compensation by Rab11b which is 91% identical to Rab11a (Welz et al.,

2014). We hypothesize that the loss of Rab11a causes a partial mislocalization of proteins necessary for ciliogenesis, but compensation by Rab11b allows for ciliation. The fact that apical accumulation of Rab11b and Rab8a is lost in Rab11a knockout and there is an increase in expression of Rab11b protein in the gut supports this notion (Knowles et al., 2015).

Rab11a in sculpting stereocilia

Recently studies in gut epithelia have revealed that Rab11a is vital for the apical protrusions of the intestinal epithelia, microvilli. With the loss of Rab11a in gut epithelia, microvilli atrophied leading to their reduction in length and width and a loss of apical specificity of microvilli (Feng et al., 2017; Knowles et al., 2015; Sobajima et al., 2014). These data clearly implicate Rab11a in the formation of apical structures. The unique apical structures, the stereocilia of the cochlea are modified microvilli that have lengthened and thickened in a precise formation on the apical surface.

Similar to the gut epithelia, loss of Rab11a in the cochlea causes a malformation in the stereocilia. Unlike reports in the gut, Rab11a stereocilia do not appear blatantly shorter or wider evident by SEM. The defect seen is in the precise patterning of the stereocilia. In late postnatal development, Rab11a loss causes a disruption in stereocilia patterning manifesting as missing or circular stereocilia bundles. In post-natal development, these phenotypes are replaced by stereocilia that are fragmented or missing sections or that are separated into 2 or more distinct misshapen stereocilia bundles. Our initial hypothesis of the cause of this phenotype was mislocalized Radixin as this protein is responsible for attaching the actin in the stereocilia to the plasma membrane and its close relative Ezrin was mislocalized in the gut (Knowles et al., 2015;

Sobajima et al., 2014). Without Radixin there is fragmentation of the stereocilia (Kitajiri et al., 2004). However, there is no difference in Radixin localization or expression in knockout stereocilia. With this no longer a possibility, we hypothesized that another actin altering protein could be affected which could cause a loss in stereocilia building at specific sites. Since we found no change in actin or beta-Spectrin marking the cuticular plate responsible for anchoring stereocilia to the cell, we concluded that this was not the cause for the defect. We noticed that this phenotype resembled the phenotype for Usher proteins, specifically Cadherin23 and Protocadherin15 mutants which lack tip links between their stereocilia leading to a splaying or fragmentation of stereocilia (Geng et al., 2013; Michel et al., 2005). We utilized Espin transfected LLC-PK1-CL4 cells to determine that Rab11a regulates Cadherin23 localization. This implicates Rab11a in the trafficking of Cadherin23 and possibly other Usher proteins responsible for adhesion between stereocilia. Additionally, these mice have profound hearing loss consistent with the Usher protein phenotype and Usher protein's function as part of the mechanotransduction machinery of the stereocilia. Sensory hair cell loss is often observed when functional loss of mechanotransduction occurs. This could mean that Rab11a regulates trafficking of tip link proteins such as Cadherin23 leading to a loss of adhesion between individual stereocilia ultimately leading to loss of sensory hair cells affecting hearing. However, it remains unclear why there is such a distinguishable difference in the phenotype between IHCs and OHCs early in development.

Recent advances in transcriptomics have highlighted the difference between inner and outer HCs. A variety of papers have recently used microarray or RNAseq to discern the difference between gene expression in IHCs and OHCs (Burns et al., 2015;

Li et al., 2016; Li et al., 2018; Liu et al., 2014; Yizhar-Barnea and Avraham, 2017). In addition, mounting evidence suggests that IHC and OHC serve different functions in the cochlea and as a consequence gene expression is different in the cell types (Men et al., 2015). We suspect that Rab11a and Rab11b are expressed at different levels in inner and outer HCs. While immunostaining cannot give an exact quantification of Rab11a in each hair cell type, it appears that the basal body pool of Rab11a is less significant in IHCs compared to OHCs (Figure 3.1). However, studies using microarray from adult cochlea found that while Rab11a mRNA was mostly equally expressed in both IHC and OHC, slightly more Rab11a was identified in IHCs (Liu et al., 2014). While the cause for the difference in phenotypes between inner and outer hair cells remains unclear, there are examples in the literature where a single mutation can cause different phenotypes in different cell types of the ear. Specifically, a mutation in SorCS2 caused OHCs to have multiple clusters of stereocilia lacking orientation while IHCs to have more stereocilia in a bundle covering a larger surface area while each individual stereocilia was shorter (Forge et al., 2017). This distinctly shows that the same genetic background gives rise to different phenotypes based on the transcriptome of the specific cell. Likewise, this could explain why in Rab11a CKO cochlea IHCs are affected to a larger degree than OHCs.

Rab11a in planar cell polarity

Several researchers have implicated the Rab11 family in planar cell polarity. *In vitro*, Rab11a marked recycling endosomes carried Celsr1 when PCP proteins were internalized and redistributed during cell divisions (Devenport et al., 2011). Additionally, Rab11 has been implicated in multiple processes in *Xenopus* embryogenesis (Kim et

al., 2012; Ossipova et al., 2015; Ossipova et al., 2014). We analyzed the localization of PCP proteins Vangl2 and Frizzled3 in Rab11a CKO cochlea and found no change in the planar or apical basal localization of these proteins. Additionally we could not discern a PCP phenotype upon knockout of Rab11a alone. This was not surprising since we believe that other Rab11 family members may be compensating for loss of Rab11a. However, when crossed with the Vangl2-looptail mutant, cochlea did show a planar cell polarity phenotype in the apical region of the cochlea that was most prominent in the most lateral outer hair cell row. There are several possibilities that could account for this phenotype. Developmentally, hair cells in the apical region of the cochlea are less mature than the hair cells at the base. Additionally, more lateral cells are more developmentally immature compared to the medial inner hair cells. This could mean that there is a developmental delay cause by loss of Rab11a. Typically the apex in the cochlea has developed stereocilia by P0 but does not in Rab11a CKO cochlea. The other possibility is that Rab11a helps link the basal body to the machinery that builds the stereocilia. In this model, Rab11a would act as an intermediate in the pericentriolar region between the kinocolia and stereocilia patterning proteins discussed previously.

Taken together our data indicates that Rab11a plays an essential role in the development of the apical structures of the sensory hair cell. Through differences in IHCs and OHCs and differences along the basilar membrane, different hair cell types and regions have a stronger or weaker penetrance of the Rab11a CKO phenotype.

Material and Methods

Mouse Strains and Animal Care

Animal care was compliant NIH guidelines and was approved by Emory University IACUC. *Rab11a* conditional knockout alleles, Vangl2-Looptail mice (Jackson Lab Jax stock #000220), and *IFT88* conditional knockout alleles were described previously (Haycraft et al., 2007; Kibar et al., 2001; Yu et al., 2014). *Rab11a*, and *IFT88* conditional alleles were inactivated via Cre-recombinase driven by Pax2Cre (Ohyama and Groves, 2004).

Reverse-Transcriptase PCR (RT-PCR)

Cochlear epithelia were dissected from embryonic day 14.5 (E14.5), E16.5, and postnatal day 0 (P0) wildtype mice and stored at -80˚Celcius until RNA isolation. RNA was isolated using RNeasy Miki Kit (Qiagen) homogenizing with QIAshredder homogenizers (Qiagen) and DNase digestion with DNase 1 recombinant, RNase-free (Roche) per manufacturer's instructions. Cochlear cDNA was made form isolated RNA using M-MLV Reverse Transcriptase (Invitrogen) with oligoDT and random primers per manufacturer's instructions. Rab11a primers were (forward) 5'-CCAGGTTGATGGGAAAACAATA-3' and (reverse) 5'-AGACATGTCATTTTCACGTCT-3' and Rab11b primers were (forward) 5'-ACGCTTCACCAGAAACGAATTC-3' and (reverse) 5'-CAGGGGACTCATCGTGGGC-3'.

MEF Cell Culture Experiment

Production of wildtype and Rab11a null mouse embryonic fibroblasts (MEFs) were described previously (gift from Nan Gao, Rutgers University, Newark, New Jersey, USA) (Yu et al., 2014). MEFs were serum starved for at least 13 hours before

staining to induce ciliation. Rescue of Rab11a-null MEFs was performed by transfecting DsRed-Rab11a plasmids using Lipofectamine2000 (Invirtogen) and standard protocols. Cells on coverslips were fixed with 4% paraformaldehyde in PBS for 15 minutes, permeabilized in 0.1%TritonX-100 in PBS (PBS-T) for 10 minutes, blocked with 10% donkey in PBS-T serum for 1 hour, and incubated with primary and secondary antibodies. Cells were scored for ciliation and analyzed as described below.

LLC-PK1-CL4 Cell Culture Experiments

LLC-PK1-CL4 cells (Gift from James Bartle, Northwestern University, Chicago, Illinois, USA) were cultured and transfected as described previously (Zheng et al., 2010). Briefly, cells were cultured at 37˚ in Minimum Essential Medium Alpha (MEM-alpha, Gibco, #12561-056) with 10% fetal bovine serum (FBS, Atlanta Biologicals #511150, 1% penicillin streptomycin (TheromFisher, #15140-122), and split (1:5 to 1:10) using standard procedures approximately every two to three days.

Before transfection, cells were passaged into a 12 well plate with a sterile #1.5 round coverslip (Fisher, #12-545-100). Once cells reached 70-80% confluence they were transfected with Lipofectamine2000 (Invitrogen #11668-019) per manufacturers instructions with the plasmids below and allowed to incubate on the cells in MEM-alpha (no FBS, no penicillin/streptomycin) for 3-4 hours. After incubation with Lipofectamine2000 cells were rinsed with complete MEM-alpha media and incubated in said media for an additional 18-24 hours. Plasmids transfected include Espin-GFP, Cdh23-Flag, Harmoninb-GFP, Espin2b (untagged) (Gifts from James Bartle, Northwestern University, Chicago, Illinois, USA) described previously (Zheng et al., 2010) and Ds-Red-HA-Rab11a and Ds-Red-HA-Rab11a-S25N (dominant negative).

Staining was achieved as described above and samples were imaged using an Olympus FV1000/TIRF microscope.

Inner Ear Dissection, Immunostaining and Imaging

Standard procedures were used for the dissection and immunostaining of inner ears. Briefly, temporal bones from E18.5 to P2 were harvested and fixed in 4% paraformaldehyde in PBS for 2 hours at room temperature, 2 hours on ice, or overnight at 4 degrees Celsius. Temporal bones were washed with PBS and stored in PBS at 4 degrees until microdissection.

The organ of Corti was microdissected and blocked in 10% donkey serum in 0.1% Triton-X-100 in PBS (10%DS in PBS-T) for 1 hour at room temperature. Organ of Corti tissue was incubated in primary antibody in 5% DS in PBS-T overnight at 4 degrees. After three five minute washes in PBS-T, tissue was incubated in secondary antibody and/or phalloidin in 5% donkey serum and PBS-T for two hours at room temperature. Tissues were again washed three times for five minutes each in PBS-T. Samples were mounted in Fluoromount-G (SouthernBiotech, #0100-01) with 1.5 coverslips and sealed.

The following primary antibodies were used: Rab11a (Cell Signaling Technology #2413,1:200), gamma-tubulin (Sigma, #T6657, 1:200), Arl13b (Tamara Caspary, Emory University, Atlanta, GA, USA; 1:1500), Vangl2 (R&D Systems #AF4815, 1:200), Fz3 (Gift from Jeremy Nathans, Johns Hopkins University, Baltimore, MD, USA, 1:500), LGN (gift from Fumio Matsuzaki, RIKEN, Kobe, Japan, 1:200), beta-spectrin (BD

Transduction Laboratories #612562, 1:200), MyosinVIIa (Proteus Bioscience Inc. #25-6790, 1:200), Radixin (Abcam #ab52495, 1:100), and E-Cadherin (Invitrogen #13-1800, 1:200).

Confocal Images were obtained using Olympus FV1000/TIRF or Zeiss LSM510 confocal microscopes. Image analysis including production of orthogonal views, Z-projections, and figures was completed in ImageJ (NIH) and Adobe Photoshop.

Scanning Electron Microscopy

Mouse organ of Corti samples were fixed in 3mM calcium chloride, 2.5% glutaraldehyde in 0.1M cacodylate and allowed to fix overnight. Samples were then rinsed with 0.1M cacodylate buffer followed by post fixed in 1% osmium tetroxide in 0.1M cacodylate for 1 hour and rinsed in deionized water. The samples were dehydrated through an ethanol series and then placed in 100% dry ethanol. The samples were placed into labeled microprous specimen capsules and loaded into the sample boat of a chilled Polaron E3000 critical point drying unit. The unit was sealed and filled with liquid CO2 under pressure. The CO2 was allowed to gently wash through the chamber and exchange for the ethanol in the tissue. When the exchange was complete, the CO2 was brought to its critical point of 1073 psi and 31°C and allowed to gently bleed away.

The dry samples were mounted on labeled SEM stubs. The stubs were coated using a Denton Vacuum Desk II sputter coater with a Gold/Palladium target. The samples were imaged at 10kV using the lower stage of a Topcon DS130 field emission scanning electron microscope and images collected using a Quartz PCI digital image collection system.

Phenotypic and statistical analysis

Stereocilia Morphology

Stereocilia morphology of IHCs and OHCs was counted and scored based on the shape of the stereocilia bundles as follows: normal stereocilia have an inversed V-shaped bundles, split stereocilia have two or more groupings of stereocilia which have split apart from one another, circular stereocilia have stereocilia that are no longer in the V shape but now in the shape of an “O”, and flat stereocilia are connected in a line but there is no vertex. For each genotype, three independent cochlea were counted and quantified noting the region and hair cell type. Statistical tests were run using a t-test for samples where two genotypes were being compared, a one-way ANOVA to compare regions in a single genotype, and a two-way ANOVA to compare between regions between genotypes. P-values ≤0.05 were considered significant.

Stereocilia Orientation

To determine stereocilia orientation, a line was drawn across the planar axis and another line was drawn to bisect the middle of the V-shape stereocilia bundle to create an angle. In the absence of properly formed stereocilia the actin devoid fonticulus, typically at the vertex of the V-shaped stereocilia bundles was used to determine the hair cell’s orientation. This angle was compared to the angle formed by the planar axis to the medial-lateral axis of the cell (90˚). Only the apex of the hair cell was counted as no apparent deviation in orientation was seen in the other regions. The hair cell row was recorded and at least 75 cells form each hair cell row was counted for each of the three samples per genotype. The most lateral row of hair cells of the apex of the cochlea was

used to statistical analysis in Oriana3. A chi-squared test was used to compare the proportion of cells deviating from 30˚ from the normal angle of 90˚ between genotypes.

Cilia Quantification.

Presence of a cilia marked by Arl13b staining was recorded and separated by IHC row and OHC row. A minimum of 300 IHCs and 1200 OHCs were counted per each region of each genotype. Statistical tests were run as described above for the stereocilia phenotype.

General Statistical Analysis and Software

Software for statistical analysis was used from www.graphpad.com/quickcalcs and a p-value of 0.05 or lower was considered significant unless otherwise noted.

Auditory brainstem response (ABR) measurement

ABR measurements were carried out within a sound attenuating booth (Shanghai Shino Acoustic Equipment Co., Ltd). Mice were anaesthetized with chloral hydrate (480 mg/kg i.p.), and then were placed onto a small animal heating pad to maintain body temperature (Automatic thermostation, BORO Zoo Co., Ltd). Subdermal needle electrodes (Rochester Electro-Medical, Inc., Lutz,FL, USA) were placed at the vertex (active, non-inverting), the left infra-auricular mastoid region (reference, inverting), and the right infra-auricular mastoid region(ground). The acoustic stimuli for ABR were produced by software SigGenRZ and the responses recorded using a TDT system controlled by BioSigRZ, digital signal processing software (Tucker-Davis Technologies, Inc., Alachua, FL, USA). Differentially recorded scalp potentials were band-pass filtered between 0.3 and 3 kHz over a 20-ms epoch. A total of 400 trials were averaged for each

stimulus condition. ABRs were elicited with digitally generated (SigGenRZ, TDT) pure tone pips presented free field via a speaker (TDT, Inc., Part MF1 2020) positioned 10 cm from the vertex. Symmetrically shaped tone bursts were 3 ms long (1 ms raised cosine on/off ramps and 1 ms plateau) and were delivered at a rate of 20 per second. Stimuli were presented at frequencies at 8 ,16,24,32 kHz and in 5 dB decrements of sound intensity from 90 dB SPL to 20 dB SPL. The ABR threshold was defined as the lowest intensity (with 5 dB resolution) capable of evoking a reproducible, visually detectable response with S/N ratio of ~1.5. Amplitudes (mV) and latencies (ms) of the initial ABR peak (wave I) were then determined at frequencies 11.3, 16 and 22.6 kHz. The analysis was carried out offline in BioSig on traces with visible waves. Latency was determined as the time from the onset of the stimulus to the peak while amplitude was measured by taking the mean of the DV of the upward and downward slopes of the peak.

Figures

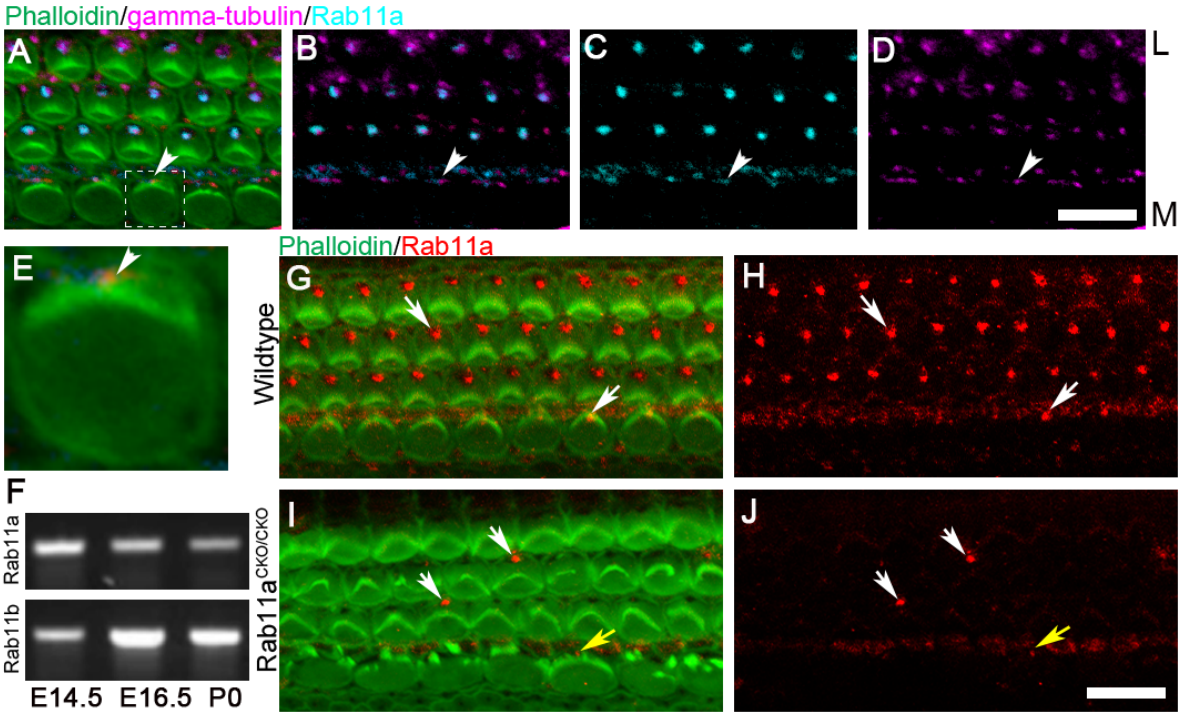

Figure 3.1. Rab11a is expressed in the organ of Corti and localized adjacent to the basal body of sensory hair cells.

(**A-E**) Cochlea from a wildtype postnatal day 0 (P0) mice stained with phalloidin (actin/stereocilia, green), gamma-tubulin (basal body, magenta), and Rab11a (cyan). In each hair cell a bundle that is composed of actin-rich stereocilia arranged in a V-shape with the basal body at the vertex of the V-shaped stereocilia bundle. The first row of hair cells at the medial (M) or center of the cochlea duct are inner hair cells, while the three rows of hair cells lateral (L) are the outer hair cells. An inner hair cell marked by a dashed box in **A** is enlarged **(E)**. The basal body of the inner hair cell is indicated by an arrowhead.

M: Medial; L: Lateral, scale bar: 10 μm.

(**F**) RT-PCR of Rab11a and Rab11b from cDNA isolated from cochlea of embryonic day 14.5 (E14.5), E16.5, and P0 mice. Rab11a and Rab11b specific primers were used as described in *Methods*.

(**G-J**) Cochleae from P2 mice containing Rab11a floxed allele (*Rab11a*$^{fl/fl}$) or *Rab11a*$^{fl/fl}$*;Pax2-Cre* (**I,J**) mice stained with Phalloidin (actin, green) and Rab11a (red). White arrows indicate Rab11a localization near the vertex of the V-shaped stereocilia bundle in the cochlea with the floxed alleles but no Cre activation (Wildtype) (**G,H**). In mice where Rab11a was conditionally knocked out by Pax2Cre (*Rab11a*$^{CKO/CKO}$), Rab11a protein staining is mostly not detectable (**I,J**). The hair cells with remaining Rab11a protein staining near the vertex of the outer hair cell stereocilia are indicated by white arrows. The yellow arrow marks an inner hair cell with an intact stereocilia bundle. Scale bar: 10μm.

Experiments for A-F were completed by Sun Kim.

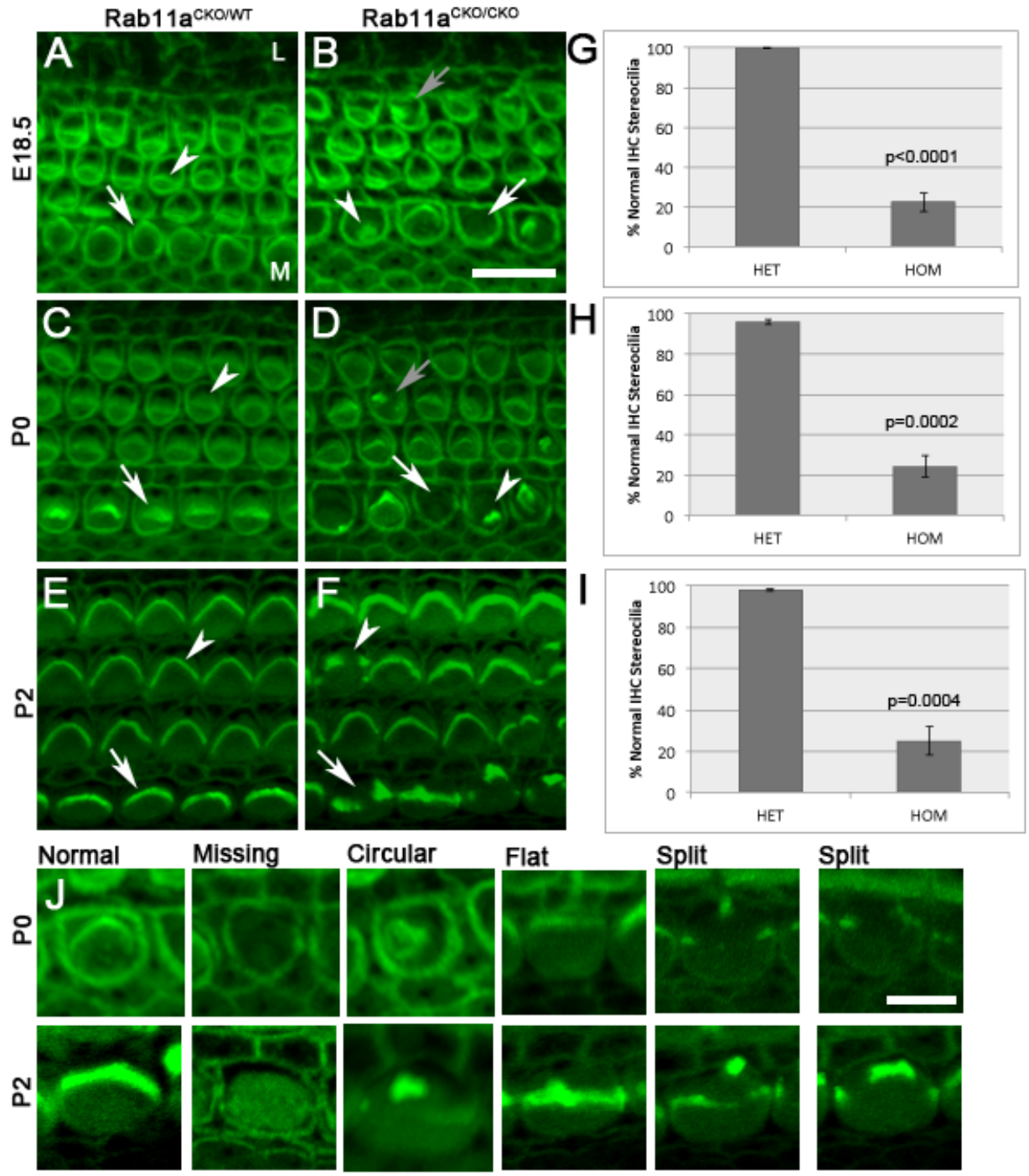
Rab11a^CKO/WT
Rab11a^CKO/CKO
A
B
L
M
E18.5
G
% Normal IHC Stereocilia
100
80
60
40
20
0
p<0.0001
HET
HOM
C
D
P0
H
p=0.0002
E
F
P2
I
p=0.0004
Normal
Missing
Circular
Flat
Split
Split
J
P0
P2

Figure 3.2. Depletion of Rab11a leads to abnormal development of hair bundles in cochlear sensory hair cells.

(**A-F**) Cochlea from Rab11a heterozygous conditional knockout (*Rab11a*$^{CKO/+}$) (**A,C,E**) and Rab11a homozygous conditional knockout (*Rab11a*$^{CKO/CKO}$) mice (**B,D,F**) at E18.5 (**A,B**), P0 (**C,D**), or P2 (**E,F**) were stained with phalloidin (actin, green) to visualize the actin-filled stereocilia bundles. In *Rab11a*$^{CKO/+}$ cochlear hair cells, stereocilia are patterned into a V-shaped hair bundle at the apical surface of both inner (arrow) and outer (arrowhead) hair cells (**A,C,E**). In *Rab11a*$^{CKO/CKO}$ cochlea, the inner and outer hair cells bundles are malformed most prominently in inner hair cells (**B,D,F**). In *Rab11a*$^{CKO/CKO}$ cochlea, there are inner hair cells with missing stereocilia (arrow), and circular or clustered stereocilia bundle (arrowhead) at E18.5 (**B**). In the outer hair cells, the stereocilia bundles are mostly normal while few malformed stereocilia bundles were observed (**B**, grey arrow). At P0 (**D**), abnormal hair bundle formation includes missing (arrow) or circular/clustered (arrowhead) in the inner hair cells and split in the outer hair cells (grew arrow). By P2 (**F**), the most frequently observed malformation of stereocilia are split hair bundles in the inner hair cells (arrow) and outer hair cells (arrowhead). Scale bar= 10 μm. M: Medial, L: Lateral

(**G-I**) Aberrant stereocilia bundle morphology of inner hair cell (IHC) was quantified and graphed as a percent of the total number of IHCs. Morphology comparison between heterozygous and homozygous *Rab11a* knockout cochleae at E18.5 (**G**), P0 (**H**), and P2 (**I**) showed a significant reduction in normal stereocilia bundle morphology in homozygous *Rab11a CKO* cochleae. Data from 3 animals at each stage for each genotype were included and averaged with error bars representing SEM.

(**J**) Example normal and abnormal (missing, circular or cluster, flat, and split) stereocilia phenotypes at P0 and P2. Scale bar= 5μm.

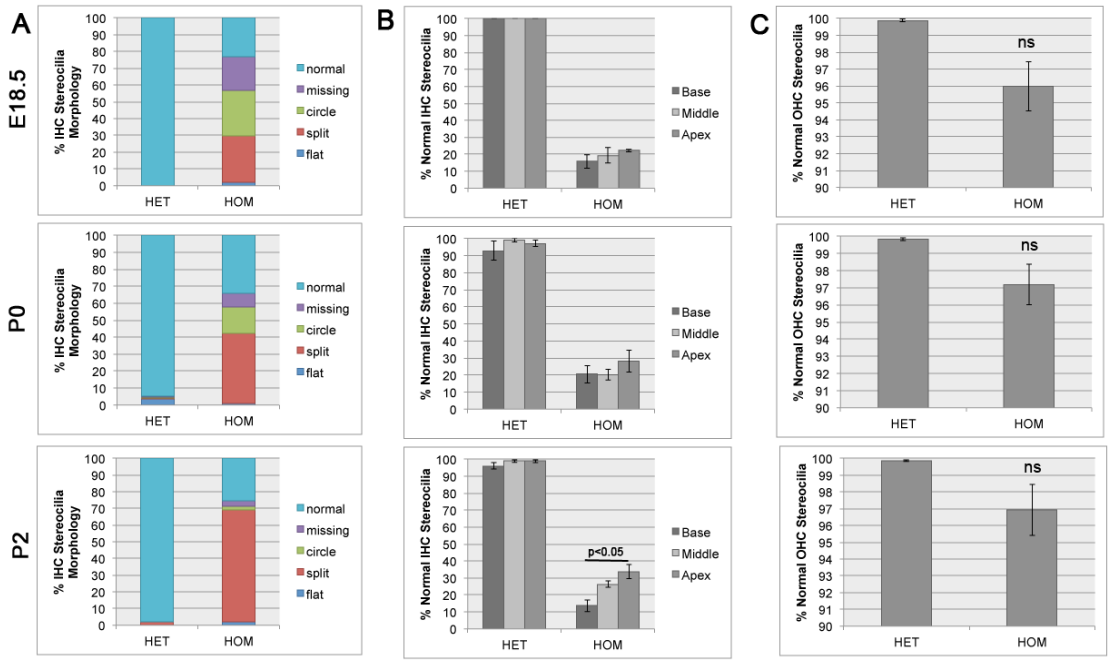

Figure 3.3. Rab11a Knockout mouse cochlear phenotype quantification

(**A**) Inner hair cell (IHC) stereocilia morphology phenotypes (normal, missing, circular, flat, or split) were counted for *Rab11a* heterozygous (HET) and homozygous (HOM) conditional knockout at E18.5, P0, and P2. Percentages of each phenotype were graphed.

(**B**) Normal IHC stereocilia bundle morphology was quantified for each region of the cochleae along the longitudinal base-apex axis of the cochlear duct. Unlike most other developmental phenotypes that often show more prominent phenotypes toward the apex of the cochlear duct, the stereocilia bundle phenotype in *Rab11a* mutants is strongest at the base of the cochleae. Average and SEM of three samples were plotted for each region, genotype, and stage. Only the P2 homozygous sample had a statistically significant difference in the % normal IHCs from the apex to base as found by a one one-way ANOVA test.

(**C**) Quantification of stereocilia morphology in outer hair cells (OHC) showed a non-significant reduction in normal stereocilia. Average and SEM of three samples for each genotype were plotted at each stage. P-values equal 0.0556 (E18.5), 0.0861 (P0), and 0.1274 (P2).

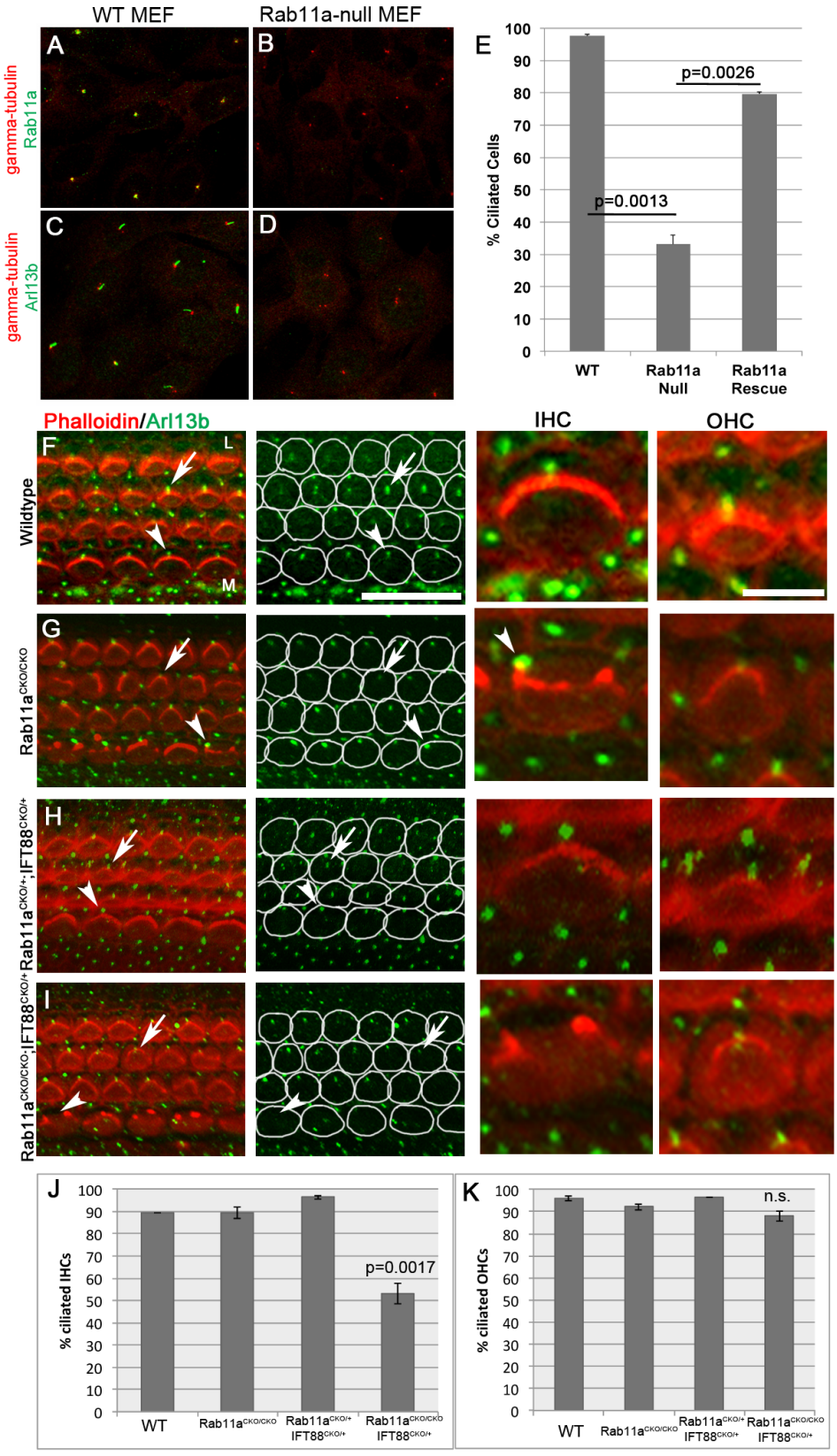
WT MEF
Rab11a-null MEF
A
B
C
D
gamma-tubulin
Rab11a
gamma-tubulin
Arl13b
E
% Ciliated Cells
p=0.0026
p=0.0013
WT
Rab11a Null
Rab11a Rescue
Phalloidin/Arl13b
IHC
OHC
F
L
M
Wildtype
G
Rab11a CKO/CKO
H
Rab11a CKO/+;IFT88 CKO/+
I
Rab11a CKO/CKO;IFT88 CKO/+
J
% ciliated IHCs
p=0.0017
WT
Rab11a CKO/CKO
Rab11a CKO/+ IFT88 CKO/+
Rab11a CKO/CKO IFT88 CKO/+
K
% ciliated OHCs
n.s.

Figure 3.4. Rab11a plays a role in ciliogenesis and works in concert with IFT88 to form the kinocilia.

(**A-E**) Wildtype (**A,C**) and Rab11a-null (**B,D**) mouse embryonic fibroblasts (MEFs) were stained with g-tubulin (basal body, red) and Rab11a (green) (**A,B**) showing the localization of Rab11a to the vicinity of the basal body region in the wild type MEFs (**A**) which is completely lost in Rab11a-null MEFs (**B**). In wildtype MEFs, ciliation was extensive as shown by the staining with a cilia marker Arl13b (**C**, green), while ciliation was lost in Rab11a-null MEFs (**D**). The percentages of cells containing a cilium from wild-type MEFs, Rab11a-null MEFs, or Rab11a-null MEFs transfected with DsRed-Rab11a (Rab11a Rescue) were quantified and plotted with the averages and SEM of three experiments (**E**). Rab11a-null MEFs showed a significant reduction in ciliation which was rescued with Ds-Red-Rab11a transfection.

(**F-I**) Cochleae from P2 wildtype (**F**), *Rab11a* homozygous conditional knockout (*Rab11a*$^{CKO/CKO}$) (**G**), Rab11a and IFT88 compounded heterozygous (*Rab11a*$^{CKO/+}$;*IFT88*$^{CKO/+}$)

(**H**), *Rab11a* homozygous conditional knockout compounded with heterozygous *IFT88* conditional knockout (*Rab11a*$^{CKO/CKO}$;*IFT88*$^{CKO/+}$) (**I**) mice were stained with phalloidin (red) and Arl13b (green) to mark the stereocilia and kinocilia, respectively. White outlines of hair cells distinguish hair cell kinocilia from supporting cell cilia. Examples of enlarged views of inner hair cells (IHC) and outer hair cells (OHC) from each respective genotype are shown. Wildtype (**F**) and *Rab11a*$^{CKO/+}$;*IFT88*$^{CKO/+}$ (**G**) cochlea maintain normal stereocilia bundle morphology with the kinocilium at the vertex of the V-shaped bundle in both inner and outer hair cells. *Rab11a*$^{CKO/CKO}$ cochlea hair

cells have the kinocilia in their inner and outer hair cells despite malformed stereocilia bundles in the outer hair cell (arrow) and inner hair cells (arrowhead) (**H**). The basal body in some inner hair cells is no longer at the lateral edge of the hair cell (arrowhead enlarged IHC) (**H**). Moreover, *Rab11a*$^{CKO/CKO}$*;IFT88*$^{CKO/+}$ cochlea maintain kinocilia in the outer hair cells (arrow) but while the kinocilium is lost in about 50% of the inner hair cells (arrowhead) (**I**).

M: Medial side of the cochlear duct, L: Lateral side of the cochlear duct, IHC: Inner Hair Cell, OHC: Outer Hair Cell. Scale bars: 20 μm and 5 μm for the left two and right two columns of images **(I-F)**, respectively.

(**J,K**)- The average and SEM of the percentages of cells containing kinocilia from IHCs (**J**) and outer hair cells (**K**) were quantifies and plotted. P-values indicate differences between *Rab11a* homozygous conditional Knockout (*Rab11a*$^{CKO/CKO}$) and *Rab11a* homozygous conditional knockout compound with an *IFT88* conditional knockout allele (*Rab11a*$^{CKO/CKO}$*;IFT88*$^{CKO/+}$) mutants. The latter has a reduced percentage of kinocilia in IHCs while there is no significant change in outer hair cell kinocilia (**K**).

Experiments, analysis and figure making for parts A-E were completed by Sun Kim.

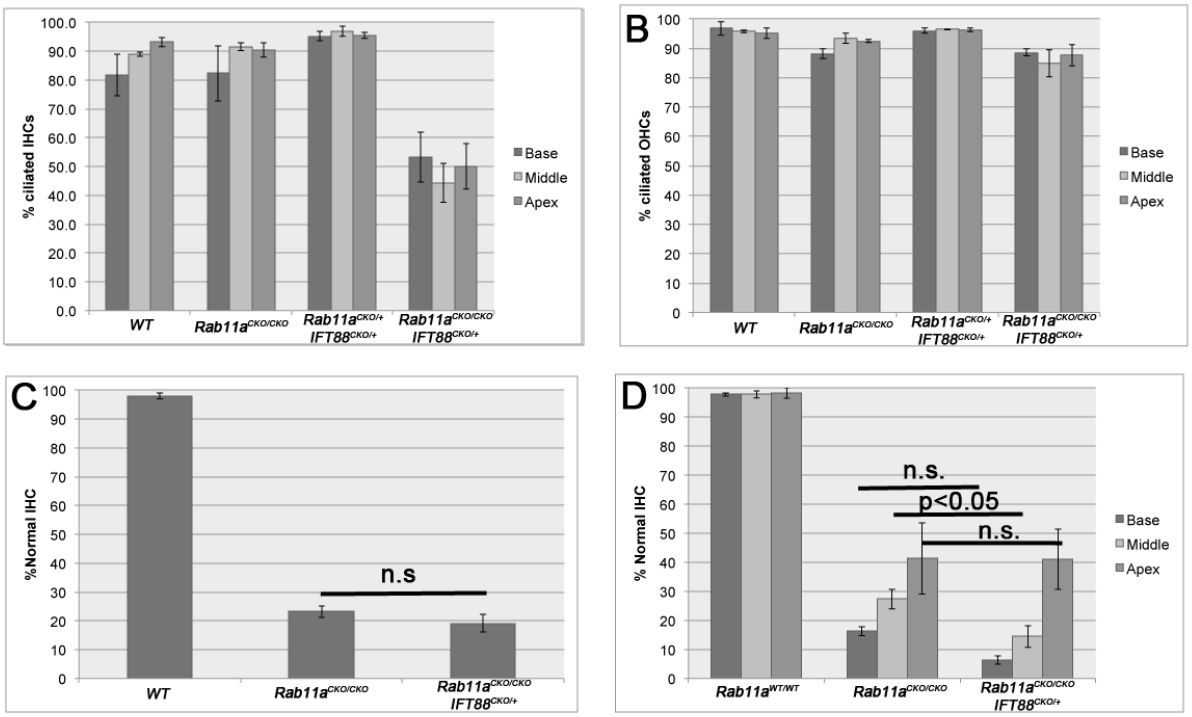

Figure 3.5. Ciliation and hair bundle morphology along the longitudinal axis of the cochlea duct in Rab11a and IFT88 mutants.

(**A**) Quantification of ciliation in IHCs in different regions, the base, middle and apex, of the cochlea in each indicated genotype. There is no statistically significant difference among the base, middle, or apex of the cochlea in any genotype. Averages and SEM of three samples were plotted. P-value a one-way repeat measure ANOVA test is 0.246 (WT), 0.699 (*Rab11a*$^{CKO/CKO}$), and 0.848 (*Rab11a*$^{CKO/?CKO}$*;IFT88*$^{CKO/+}$).

(**B**) Quantification of ciliation in OHCs in different regions, the base, middle, and apex, of the cochlea in each indicated genotype. There is no statistically significant difference among the base, middle, or apex of the cochlea. Averages and SEM of th ree samples were plotted. P-value of a one-way repeat measure ANOVA test is 0.76 (WT), 0.15 (*Rab11a*$^{CKO/CKO}$), and 0.76 (*Rab11a*$^{CKO/?CKO}$*;IFT88*$^{CKO/+}$).

(**C**) Quantification of the morphology of inner hair cell stereocilia bundle of wildtype, *Rab11a* homozygous knockouts (*Rab11a*$^{CKO/CKO}$) and *Rab11a*$^{CKO/CKO}$*;IFT88*$^{CKO/+}$ mutant cochleae. The averages and SEM of three samples from each genotype were plotted. P-value between *Rab11a*$^{CKO/CKO}$ and *Rab11a*$^{CKO/CKO}$*;IFT88*$^{CKO/+}$ is not significant despite a reduction in ciliation in the IHCs of the latter (p=0.3382).

(**D**) Quantification of the morphology of inner hair cell stereocilia bundle of wildtype, *Rab11a* homozygous knockouts (*Rab11a*$^{CKO/CKO}$) and *Rab11a*$^{CKO/CKO}$*;IFT88*$^{CKO/+}$ mutant cochleae at different regions of the cochlea, the base, middle and apex. Analysis of the different regions of the cochlea did revealed a reduction in normal stereocilia bundle morphology between *Rab11a*$^{CKO/CKO}$ and *Rab11a*$^{CKO/CKO}$*;IFT88*$^{CKO/+}$ that is only significant in the middle region of the cochlea between *Rab11a*$^{CKO/CKO}$ and *Rab11a*$^{CKO/CKO}$*;IFT88*$^{CKO/+}$ that is only significant in the middle region of the cochlea. A two-way ANOVA was used and found a p-value of <0.0001 between genotypes and 0.0013 between regions. In both homozygous and compound mutants the base is significantly different than the apex and the middle is significantly different than the apex. The base and middle are not significantly different. Only the middle region of the homozygous and compound mutants was significantly different.

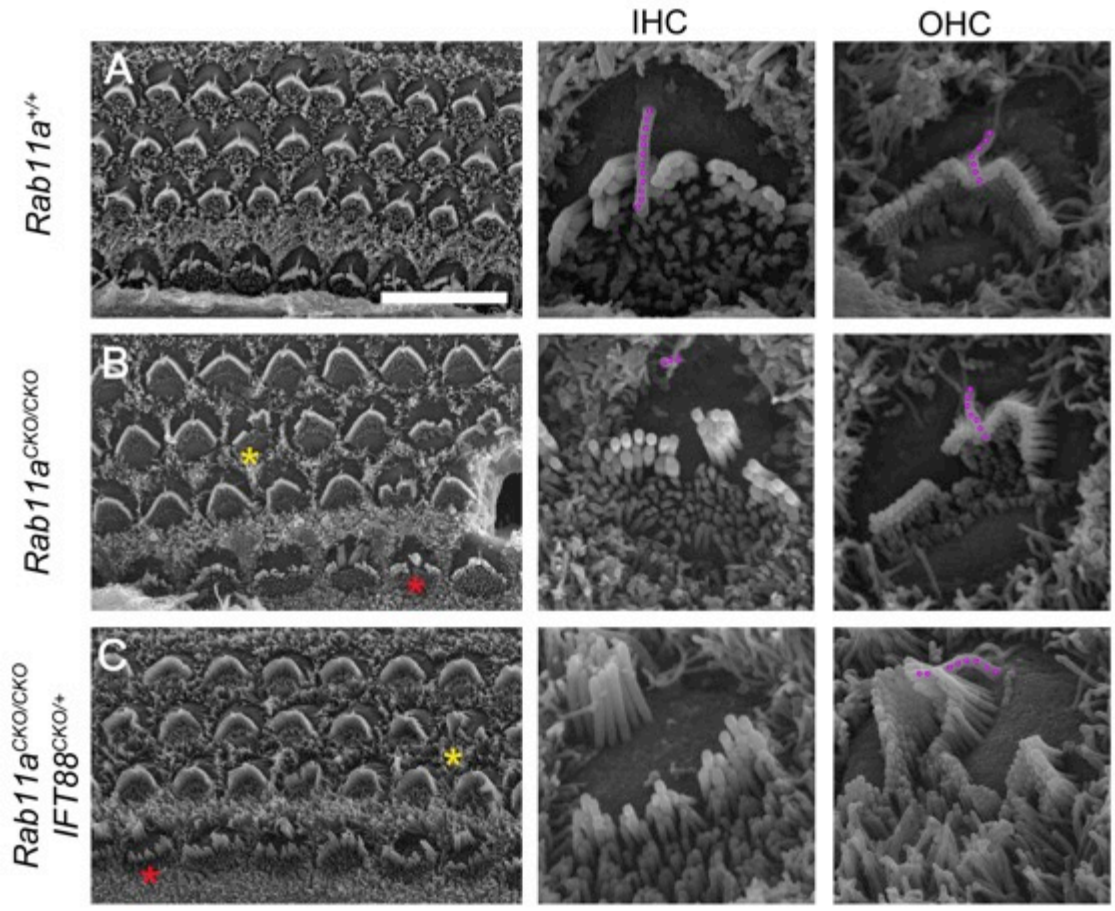

Figure 3.6. Abnormalities in the hair bundles of Rab11a mutants under scanning electronic microscope

(**A-C**) Scanning electron micrographs (SEM) of wildtype (**A**) *Rab11a* homozygous *Rab11a*$^{CKO/CKO}$ (**B**) and *Rab11a*$^{CKO/CKO}$*;IFT88*$^{CKO/+}$ mutant cochlea (**C**) at P2. The right two columns are IHCs and OHCs at a 4x magnification of the images in the left column. The IHC or OHC shown in the larger magnifications are marked with red and yellow astricks, respectively.

(**A**) Wildtype cochleae have cohesive inner hair cell (IHC) bundles and outer hair cell (OHC) bundles in the shape of a V with a kinocilium (magenta colored) at the vertex of each stereocilia bundle. They have the characteristic “bare zone” lateral to the

stereocilia with microvilli medially. OHC are further along in development and their microvilli are starting to retract.

(**B**) *Rab11a*$^{CKO/CKO}$ IHC stereocilia bundles are not formed properly. They are split (arrow, **E**) and typically have a kinocilium (magenta colored), which is less discerning due to the change in its length and its mislocalization. Some abnormal OHC bundles were also observed. Kinocilia are present in most cells (magenta colored). The bare zone and microvilli enriched regions lateral and medial to the stereocilia are present.

(**C**) *Rab11a*$^{CKO/CKO}$*;IFT88*$^{CKO/+}$ cochleae display deformed stereocilia and lack the kinocilium in IHCs. Some hair bundle in the outer hair cells are also misshapened and but retain their kinocilia (magenta colored).

IHC: Inner Hair Cell; OHC: Outer Hair Cell. Scale bar= 10 μm

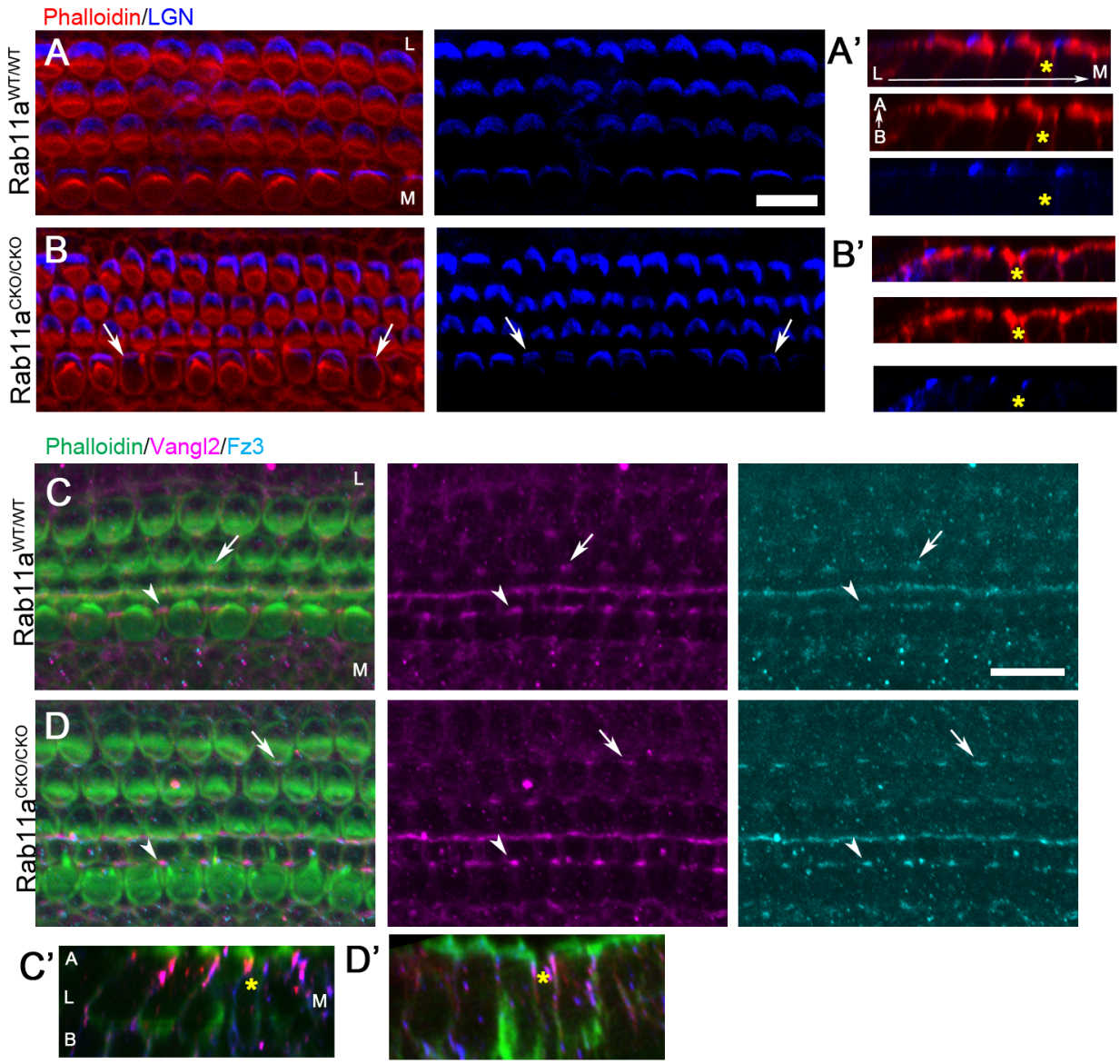

Figure3.7. Apical protein partitioning along the planar cell polarity axis is not altered in Rab11a mutants.

(**A,B**) Wildtype (**A**) and *Rab11a* homozygous conditional knockout (*Rab11a*$^{CKO/CKO}$) (**B**) cochlea at P0 was stained with phalloidin (stereocilia, red) and LGN (bare zone, blue). In the wildtype cochlea LGN marks the bare zone lateral to the stereocilia. In the *Rab11a* homozygous conditional knockout cochlea despite the loss of IHC stereocilia

and stereocilia cohesion, LGN still forms on the lateral edge of the hair cell (arrow). Orthogonal views show that LGN retains its localization at the apical surface of the hair cells in both Wildtype (**A'**) and homozygous knockout (**B'**) hair cells. A yellow asterisk marks the pillar supporting cells between the inner and outer hair cell rows.

(**C,D**) Wildtype (**C**) and *Rab11a* homozygous conditional knockout (**D**) cochlea at P0 were stained with phalloidin (green), Vangl2 (magenta), and Frizzled3 (cyan). In both the wildtype (**C**) *Rab11a* knockout (**D**) partitioning of planar cell polarity proteins Vangl2 and Frizzled3 are localized to the medial edge of hair cells where they contact supporting cells in the outer hair cells (arrow) and on the lateral sides of the supporting cells intercallated with the inner hair cells (arrowhead). Orthoganal view of Wildtype (**C'**) and Rab11a Knockout (**D'**) have Vangl2 and Frizzled localized to the apical part of the hair cells.

Scale Bars= 10μm, L: Lateral, M: Medial, A: apical, B: basal

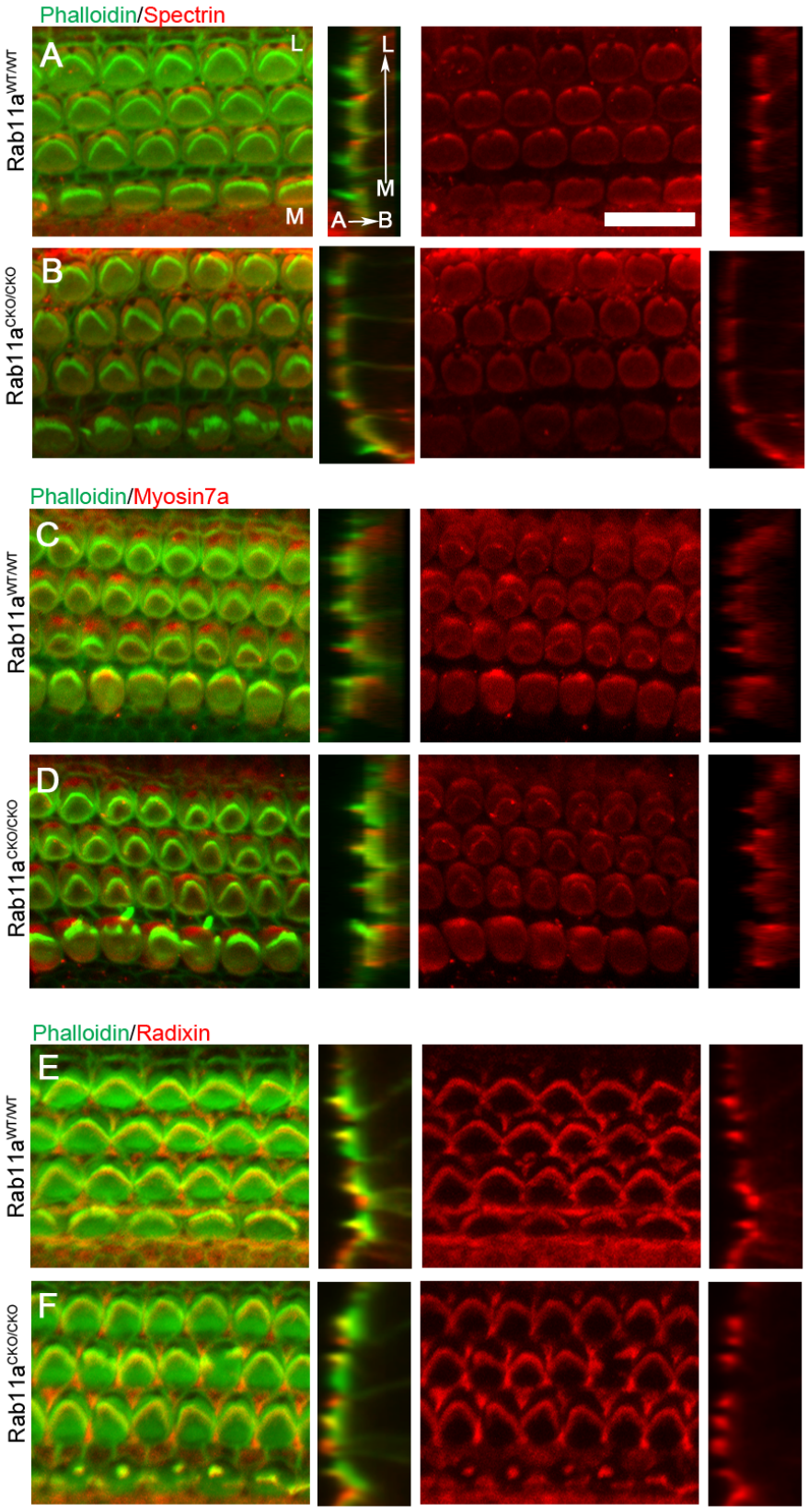
Phalloidin/Spectrin
Rab11a WT/WT
Rab11a CKO/CKO
A
B
L
M
A→B
Phalloidin/Myosin7a
C
D
Phalloidin/Radixin
E
F

Figure 3.8. Location of stereocilia architecture components is not altered by loss of Rab11a.

(**A-F**) Wildtype (**A,C,E**) and *Rab11a* homozygous conditional knockout (**B,D,F**) cochlea at P2 were stained with phalloidin (stereocilia, green) and beta-Spectrin (cuticular plate, red) (**A,B**), MyosinVIIa (hair cell motor protein, red) (**C,D**), and Radixin (actin-to-membrane-linking protein, red) (**E,F**). All proteins were localized to the apical surface in both WT and Rab11a homozygous samples. Orthogonal views (**A'-F'**) show that normal apical localization is retained for each protein. Scale bar= 10μm, L: Lateral, M: Medial, A: apical, B: basal

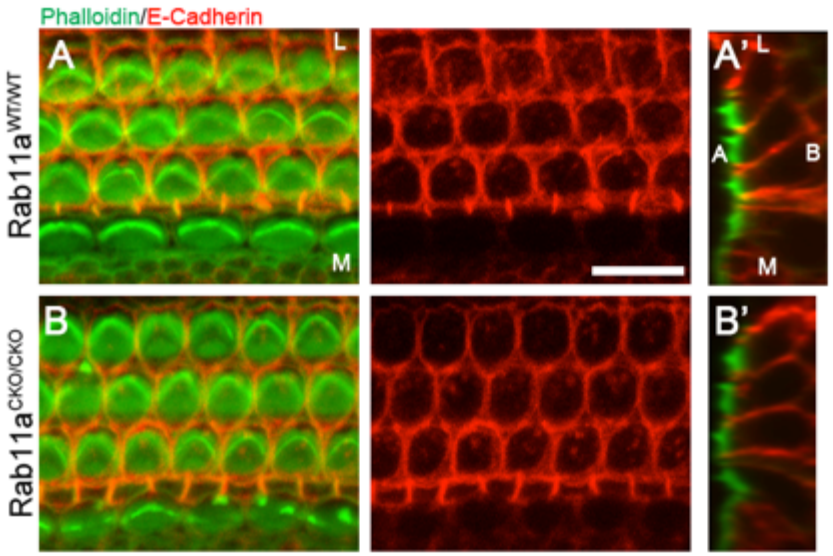

Figure 3.9. Loss of Rab11a does not alter E-Cadherin localization in the cochlea

(**A-B**) Wildtype (**A**) and Rab11a homozygous conditional knockout (**B**) cochlea at P2 were stained with phalloidin and E-Cadherin. (**A**) E-Cadherin is expressed in the outer hair cells and localized to the cell membrane in wildtype cochlea. (**B**) Knockout of Rab11a does not alter its expression of E-Cadherin in the OHCs and remains absent in inner hair cells. (**A',B'**) Orthogonal views of both wildtype (**A'**) and Rab11a Knockout (**B'**) show basolateral localization of E-Cadherin in OHCs but is absent from IHCs. Scale Bar=10μm, A: Apical, B: Basal, M: Medial, L: Lateral

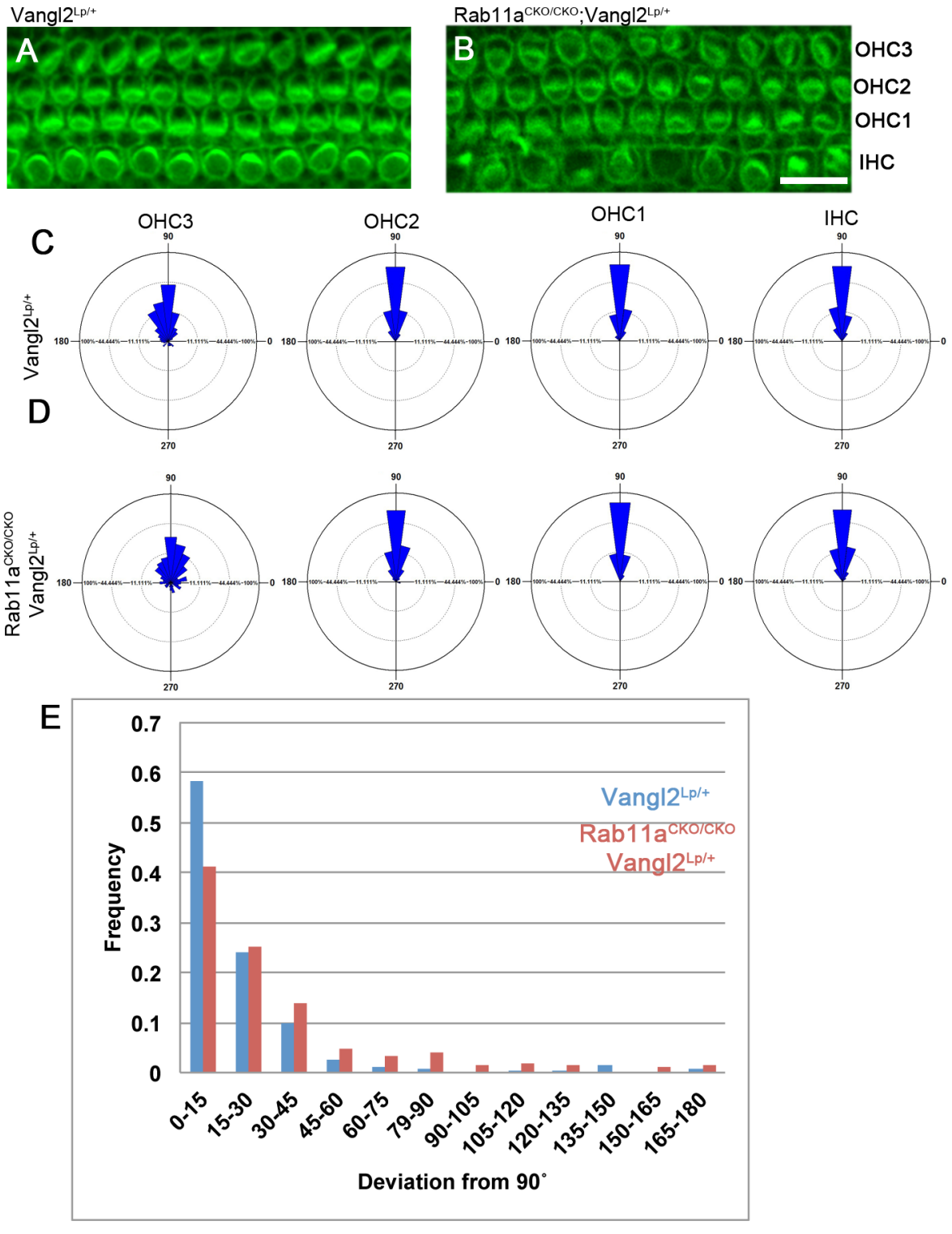
Vangl2Lp/+
Rab11aCKO/CKO;Vangl2Lp/+
A
B
OHC3
OHC2
OHC1
IHC
C
D
OHC3
OHC2
OHC1
IHC
Vangl2Lp/+
Rab11aCKO/CKO Vangl2Lp/+
90
180
0
270
E
Frequency
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Vangl2Lp/+
Rab11aCKO/CKO
Vangl2Lp/+
0-15
15-30
30-45
45-60
60-75
79-90
90-105
105-120
120-135
135-150
150-165
165-180
Deviation from 90˚

Figure 3.10. Rab11a regulates hair cell polarity in immature hair cells

(A,B) The apical region of the cochlea in Vangl2-Looptail heterozygote controls (*Vangl2*$^{Lp/+}$) and *Rab11a* homozygous knockout with Vangl2-Looptail heterozygous (*Rab11a*$^{CKO/CKO}$*;Vangl2*$^{Lp/+}$) were stained with phalloidin (stereocilia, green) to visualize hair cell polarity along the planar axis.

(C,D) Oriana graphs showing the distribution of hair cell orientation of *Vangl2*$^{Lp/+}$ (**C**) and *Rab11a*$^{CKO/CKO}$*;Vangl2*$^{Lp/+}$ (**D**). There was a decrease in oriented hair cells in the outermost HC row (OHC3) ($p<0.0001$) and less severely in OHC row 2 ($p=0.0204$) while othr HC row orientation remained unchanged.

(E) Stereocilia orientation as a function of deviation form 90˚ was plotted to show the difference in distribution of hair cell orientation between *Vangl2*$^{Lp/+}$ and *Rab11a*$^{CKO/CKO}$*;Vangl2*$^{Lp/+}$.

Scale bar= 10μm IHC: Inner Hair Cell, OHC Outer Hair Cell

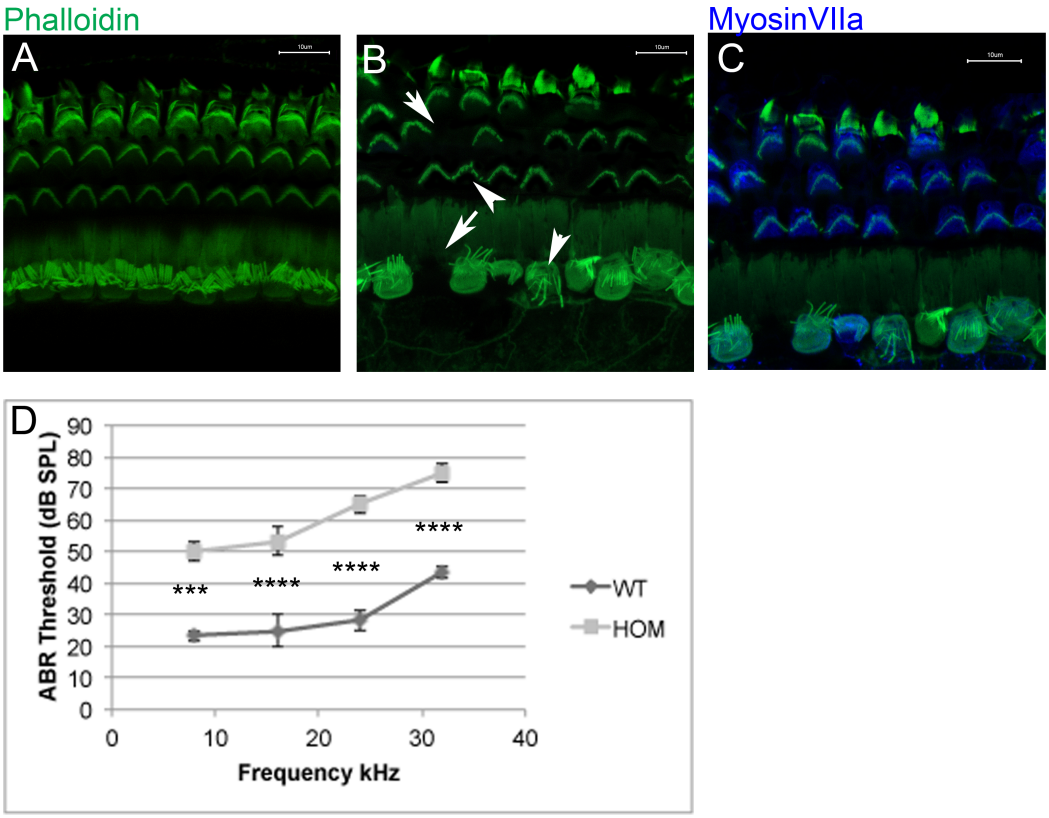

Figure 11.3. Rab11a loss causes hair cell degeneration and hearing loss.

(A-C) Cochlea from 4 week old wildtype (**A**) and Rab11a CKO (**B,C**) mice were stained with phalloidin (green) to reveal stereocilia. In wildtype outer hair cells have neat V-shaped stereocilia bundles and inner hair cells with a characteristic U shaped bundle with longer stereocilia (**A**). Rab11a CKO cochlea have missing hair cells (arrows) and stereocilia that are less organized and fragmented (arrowheads) in both inner and outer hair cell rows. Rab11a CKO cochlea were stained with MyosinVIIa antibody to visualize hair cells (**C**).

Scale Bar: 10 μm

(D) Four week old control (WT) and Rab11a CKO (HOM) mice were subject to Auditory Brainstem Response (ABR) threshold test. A two-way ANOVA with *post hoc* Sidak's multiple comparison test suggests significant change decrease in ABR threshold was

observed in Rab11a CKO mice indicating significant hearing loss. ***p<0.001, ****p<0.0001

Experiments and analysis for this figure were completed by DongDong Ren.

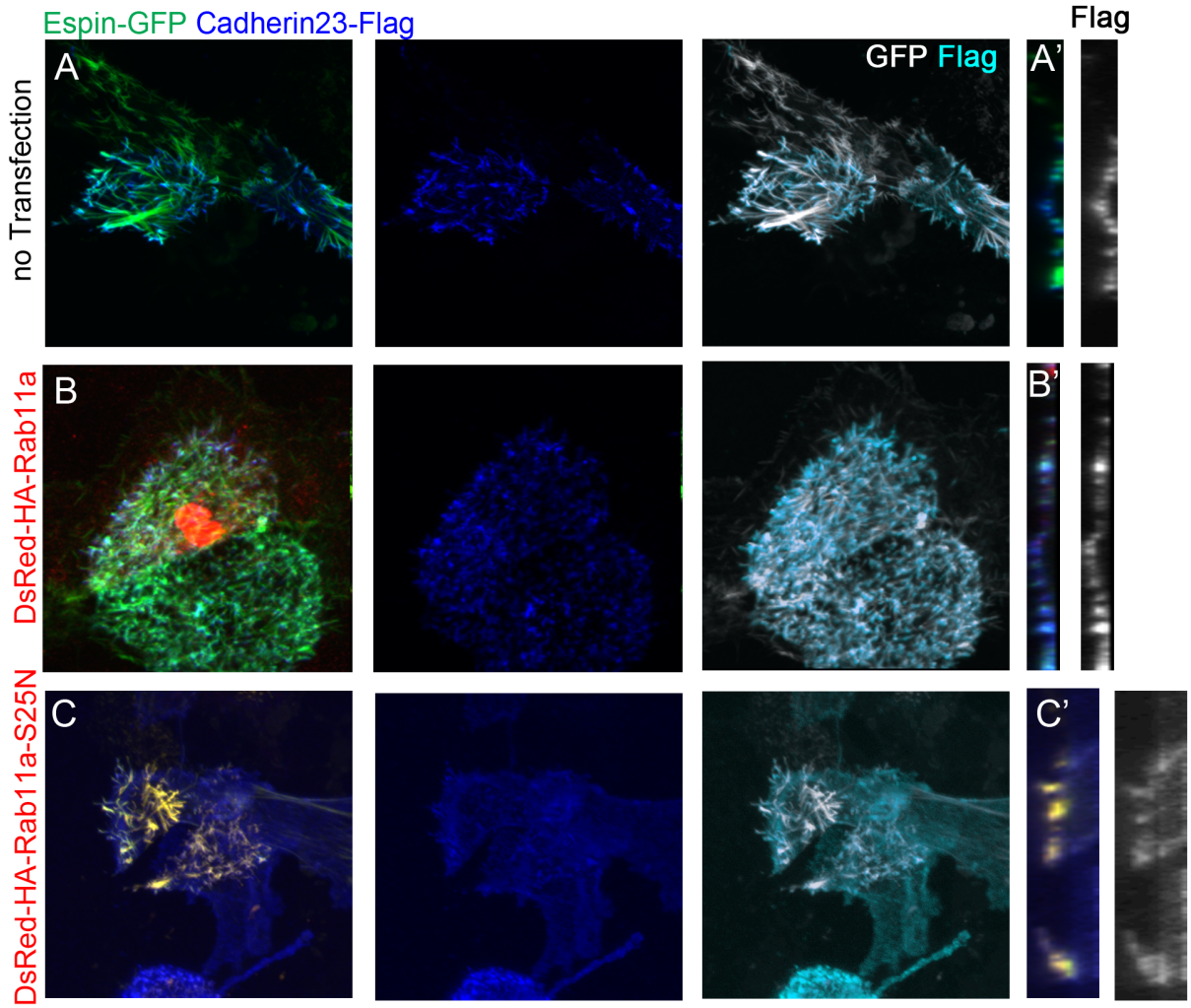

Figure 3.12. Rab11a is responsible for localization of Cadherin23 in elongated microvilli.

(A-C) Cultured LLC-PK1-CL4 cells with naturally occurring microvilli were transfected with Espin-GFP (green) to elongate the microvilli to resemble stereocilia. Localization of transfected Cadherin23-Flag (blue) was localized to the microvilli in cells not transfected (**A**) and cells transfected with wildtype (DsRed-HA-Rab11a) (**B**) and localized only apically (**A'-B'**). In dominant negative DsRed-HA-Rab11a-S25N transfected cells Cadherin23-Flag was localized throughout the cell (**C**) both apically and basally (**C'**).

A’-C’ indicates orthogonal views with Cadherin-Flag expression indicated in the second panel. Apical on the left, Basal on the right.

Chapter 4: *Ankrd6* Regulates Planar Cell Polarity In The Mammalian Organ Of Corti

This chapter was adapted from from the publication below and focuses on the Ankrd6 in the planar cell polarity pathway in mammals.

Jones C, Qian D, Kim SM, Li S, Ren D, **Knapp L**, Sprinzak D, Avraham K, Fumio M, Chi F, Chen P (2014). *Ankrd6* is a mammalian functional homolog of *Drosophila* planar cell polarity gene *diego* and regulates coordinated cellular orientation in the mouse inner ear. Developmental Biology.

Introduction

Planar cell polarity (PCP) regulates the coordination of cellular structures between neighboring cells across the plane of a tissue. The mammalian organ of Corti contains sensory hair cells interdigitated with supporting cells where PCP coordinates intrinsic polarity in the form of kinocilia and stereociliary bundle. In *Drosophila*, where PCP proteins were originally identified, transmembrane proteins Van Gogh (Vang- mammalian homolog Vangl2), Frizzled (Fz), Flamingo (Fmi- mammalian homolog Celsr1) and their associated proteins Dishelved (Dsh- Dvl in mammals), Prickle (Pk), and Diego (Dgo) are planar polarized into unique microdomains across the apical membrane to regulate PCP processes including wing hair orientation. Likewise, in the mammalian organ of Corti, disruption of PCP proteins including Vangl2, Fz, Dvl, and Celsr1 disrupts the coordinated orientation of the kinocilia and stereocilia in the sensory hair cells (Curtin et al., 2003; Montcouquiol et al., 2003; Wang et al., 2006).

Despite the identification of many mammalian homologs of the PCP proteins originally found in *Drosophila*, several PCP associated proteins have not been identified. One such protein, Dgo, has a close homolog called Ankrd6 (also called Diversin). In *Drosophilia*, Dgo polarizes to the Fz-Dsh complex and through this interaction restricts Pk to the Vang side of the cell influencing actin nucleation activity causing the hair of the wing cell to form (Das et al., 2004; Jenny et al., 2005). In vertibrates, Ankrd6 has been implicated in gastrulation during heart formation in zebrafish and regulates the basal body and polarity of cilia in *Xenopus* through regulation of Wnt signaling (Itoh et al., 2009; Itoh and Sokol, 2011; Moeller et al., 2006; Schwarz-Romond et al., 2002; Yasunaga et al., 2011). Mouse Ankrd6 (mAnkrd6)

overexpression in the *Drosophila* eye perturbs PCP (Moeller et al., 2006). Additionally, mAnkrd6 ectopic expression causes perturbations in PCP and rescues loss of Diego in the wing indicating that Ankrd6 is a functional homolog of Diego (Jones et al., 2014).

Here we investigate the role of Ankrd6 in planar cell polarity in the mouse cochlea. Ankrd6 is asymmetrically localized to the cell membrane similarly to other PCP proteins. Additionally knockout of Ankrd6 disrupts planar cell polarity in the cochlea without disrupting the morphogenesis of the supporting cells of the cochlea.

Results

Ankrd6 is asymmetrically polarized in the cochlea

The organ of Corti displays coordinated polarity across the plane of the tissue. In each sensory hair cell, actin based stereocilia form in a V-shaped pattern in graded rows with the tallest row at the lateral side of the stereocilia bundle. During development, the vertex of the V-shaped bundle is adorned with a true cilia termed the kinocilia. This intrinsic polarity of each hair cell is coordinated by the PCP pathway whose components are polarized on the medial or lateral side of the hair cells and supporting cells.

Antibody staining against Ankrd6 showed polarized localization to the cell junction of the medial hair cell between hair cell-supporting cell boundaries and between supporting cells (Figure 4.1A-B,B'). The core PCP protein Vangl2 appears to be localized to the same cellular boundary between hair cell and supporting cell (Figure 4.1C). However, it appears that the proteins are in different cells with Vangl2 localized to the supporting cell and Ankrd6 localized in the hair cell (Figure 4.1C'). Additionally Ankrd6 also appears to be localized to the medial side of supporting cells as seen in

cells labeled 1 and 2 and breaks at the cell boundary whereas the line does not extend throughout supporting cell 3 (Figure 4.1B'').

Ankrd6 interacts genetically with Vangl2 to coordinate PCP in the organ of Corti

The polarized localization of Ankrd6 positions it as a potential regulator of PCP in the organ of Corti. To investigate this possibility, we analyzed Ankrd6 null mutants from E18.5 to P10 to determine in they had any overt PCP defects. At P0 Ankrd6 wild-type, heterozygous, and homozygous null cochlea showed no difference in orientation in the sensory hair cells (Figure 4.2A-B,E-H, quantified M). At E18.5, earlier in hair cell development, we looked at the apex of the cochlea where cells are less mature than the base. In these cells Ankrd6 null cochlea have normal cellular orientation (Figure 4.2N-O). We analyzed LGN localization, an apical compartmentalization protein localized lateral to stereocilia thought to bridge polarity signals between cells to the intrinsic polarity within cells (Tarchini et al., 2013). At E18 there was no difference between Ankrd6 null and littermate control LGN staining (Figure 4.2P-Q). Since supporting cells also express Ankrd6 in a polarized fashion we analyzed the pharyngeal processes of supporting cells at P10 but saw no defect in Ankrd6 null when compared to the control in any region of the cochlea (Figure 4.3).

We further examined Ankrd6 null cochlea to determine if it had a genetic interaction with core PCP protein Vangl2. Homozygous looptail mutants loose coordinated polarity while heterozygote retain coordinated polarity in the organ of Corti (Montcouquiol et al., 2003). While double heterozygotes do not show a phenotype, *Ankrd6*$^{-/-}$*;Vangl2*$^{Lp/+}$ cochlea show a loss of coordinated polarity specifically in their outer most hair cell row (Figure 4.2I-L, quantified M). While 29.2% of outermost hair cells

were disoriented beyond 30˚ in Ankrd6$^{-/-}$;Vangl2$^{Lp/+}$ cochlea, 0.9%, 0.3% and 1.7% of the outer hair cell row was misoriented in wildtype, *Ankrd6$^{-/+}$;Vangl2$^{Lp/+}$*, and *Ankrd6$^{-/-}$* cochlea respectively. Typically the outer most hair cell row is affected the most significantly in PCP mutants. This genetic interaction with core PCP protein Vangl2 further positions Ankrd6 as a PCP associated protein and a mammalian Diego homolog.

Discussion

Ankrd6 as a PCP protein in the mammalian organ of Corti

Ankrd6 is a functional homolog to Dgo able to rescue loss of Dgo in the *Drosophila* eye and wing (Jones et al., 2014). The localization of Ankrd6 within the mouse organ of Corti further positions it as a regulator of PCP. Ankrd6 is localized to the medial hair cell and supporting cell (Figure 4.1). The localization of Ankrd6 in relation to Vangl2 and other PCP proteins is consistent with what is seen in the *Drosophila* PCP model. There, Vangl2 is localized to one side of the cell while Dgo and its transmembrane interactor Fz are localized to the opposite cellular contact. Likewise, it appears that Vangl2 and Ankrd6 occupy opposite cellular membranes in the organ of Cort.

Ankrd6 is dispensable for mouse development and does not cause any PCP defects in the organ of Corti when depleted. However, a genetic interaction with core PCP protein Vangl2 was observed when Ankrd6 null mice were compounded with the looptail mutant. Despite the need for the PCP pathway to be weakened by partial Vangl2 depletion for Ankrd6 to display a PCP phenotype, this is not uncommon for PCP associated proteins. Together these data undoubtedly implicate that Ankrd6 is the

mammalian homolog for Dgo and regulates vertebrate planar cell polarity in the organ of Corti.

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. The following mouse strain was obtained from the Jackson Laboratories: LPT/Le (Vangl2-Lp) (Kibar et al., 2001). Ankrd6 knockout mice were generated as described in Jones et al., 2014.

Inner ear sensory epithelia preparation and antibody immunostaining

Standard procedures were used to isolate and prepare whole mount inner ear sensory epithelia (Wang et al., 2005). The primary antibody used in this study were raised against Ankrd6 (described in Jones et al, 2014, 1:800). In addition, Rhodamine- or Alexa-Fluor-488 conjugated phalloidin (Invitrogen, 1:1000) were used for staining the actin-rich structures such as stereocilia, the cuticular plate of the hair cell, and the cortex of cells. For image acquisition the following microscopes were used: Olympus SZX12 upright microscope, Olympus Fluoroview FV-1000 confocal microscope, and Zeiss LSM510 confocal microscope.

Analyses of stereociliary bundle orientation and morphology

The V-shaped hair bundle orientation was determined by drawing a line from the position of the kinocilium through the middle of the V-shaped stereocilia (bisecting line). We defined the angle of orientation as the angle formed between the bisecting line and the line parallel to the medial to lateral axis of the cochlear duct. In wild-type animals,

this angle is close to 0˚. Each row of hair cells was divided into three groups according to its position along the longitudinal axis of the cochlea: base, middle, and apex. Due to the differentiation gradient within the single cochlea during development, hair cells in the apex region are less developed. Only hair cells from the base and middle regions of the cochleae were included for polarity quantification. At least 25 hair cells in each row in each region were quantified for each sample, and at least three animals per genotype were analyzed. The distribution of angles along the length of the cochlear duct was plotted using Oriana3. Cells that had a central fonticulus and/or circular stereocilia were classified as having the maximum deviation from the normal distribution, namely 180˚. Data are presented as means. Statistical significance was analyzed by Chi-square analysis and Mardia Watson Wheeler tests using Oriana3.

Figures

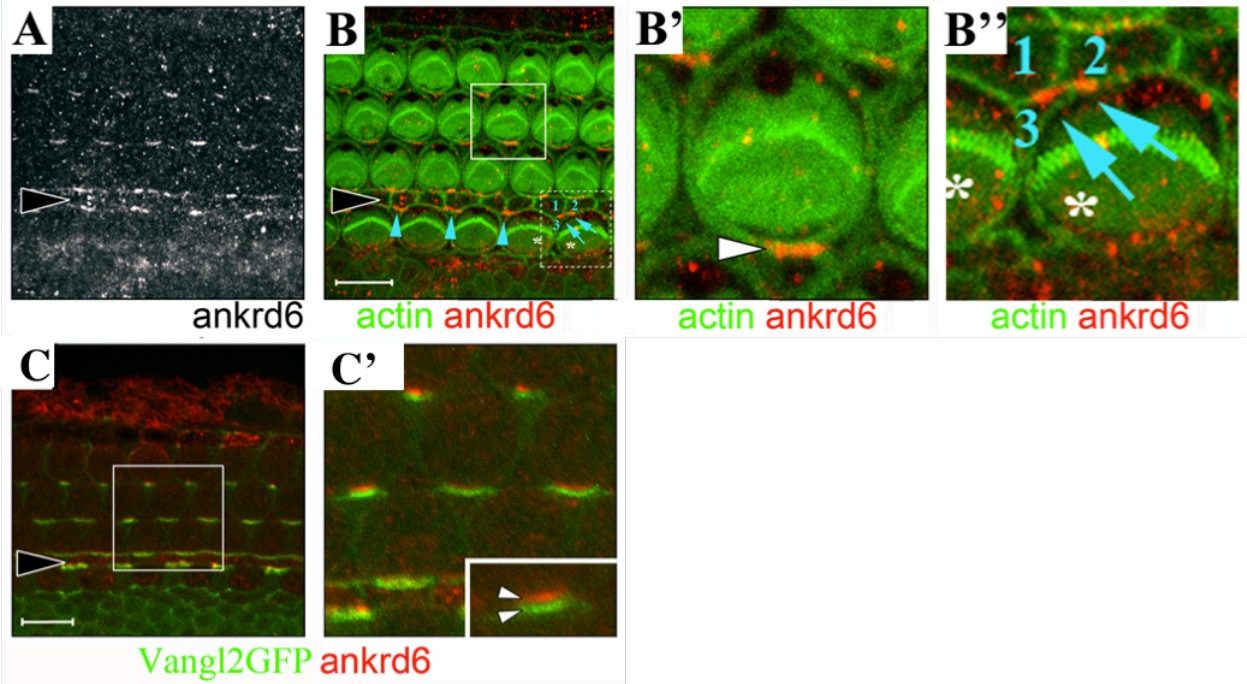

Fig. 4.1. Ankrd6 protein shows asymmetric distribution along the PCP axis in the organ of Corti characteristic of core PCP proteins.

(A-B) Confocal images of P1 wildtype cochlear whole mounts show actin (green) and Ankrd6 (A, white; B, red). Actin staining depicts stereocilia bundle orientation and cell outline. Boxes in B represent the area enlarged in B' and B''. Black arrowheads indicate the supporting cell region. Ankrd6 is observed asymmetrically at the boundaries between hair cell and supporting cells (B', white arrowheads) and boundaries between supporting cells (B, B'', blue arrowheads/arrows).

Scale bars: 10μm

(C) Confocal images of P2 cochlea whole mounts from mice carrying GFP-Vangl2 transgene (green) were stained with an antibody against Ankrd6 (red). Ankrd6 is localized to the same cellular boundary as Vangl2 but does not overlap (C', whit arrowheads). The box in C represents the area enlarged in C'.

Scale bars: 10 μm.

This figure was modified from a figure published in Jones et al., 2014. Figure prepared by C. Jones.

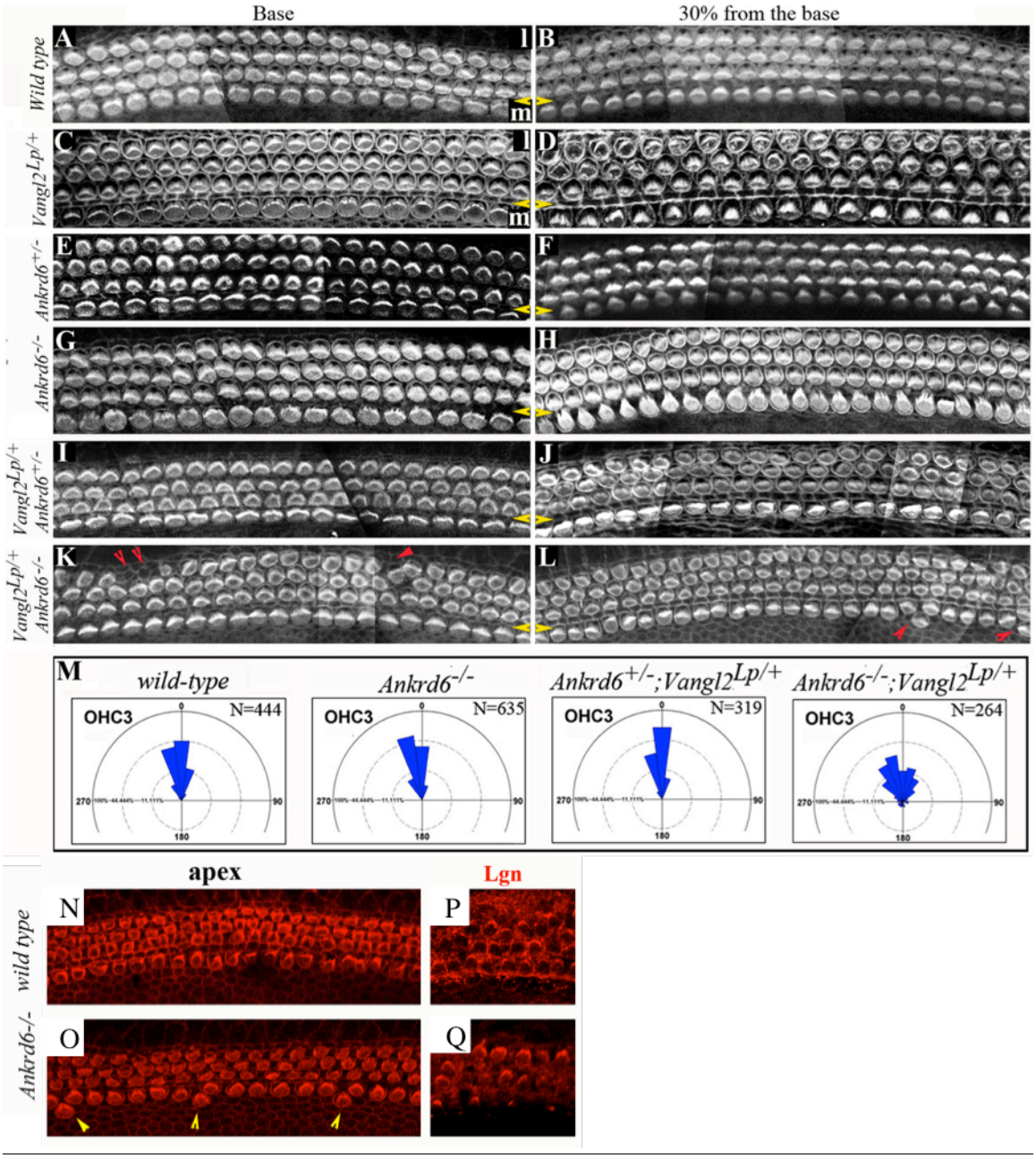

Fig. 4.2. Ankrd6 interacts with PCP gene Vangl2 to regulate the precise orientation and patterning of hair cells in the cochlea.

(A-L) Confocal images of P0 cochlear mounts isolated from wild-type (A, B), Vangl2Lp/+ (C, D), Ankrd6+/- (E, F), Ankrd6-/- (G, H), Vangl2Lp/+; Ankrd6+/- (I, J), and Vangl2Lp/+; Ankrd6-/- (K, L) mutant mice were stained for phalloidin to visualize the stereociliary

bundles and the cortical outline of hair cells. Yellow arrowheads (A-L) mark the supporting cell region that separates the out hair rows from the inner hair cell row. Red arrowheads indicate patterning abnormalities (K, L). The images were taken from the base and 30% from the base of each cochlea. m: medial side of the cochlea; l: lateral side of the cochlea.

(M) The orientation of the last row of hair cells within the region 30% from the base was measured and plotted for the genotypes indicated.

(N-O) Cochlea whole mounts were prepared from E18.5 wild-type (N) and Ankrd6-/- (O) embryos and visualized with phalloidin to see stereocilia bundles. Hair cells mature along the longitudinal gradient meaning the apex of the cochlea house the least nature hair cells. At these more immature hair cells PCP is not apparently affected in Ankrd6 embryos. Yellow arrowheads (O) mark imperfect cellular patterning in the Ankrd6-/- cochlea.

(P,Q) Cochlea whole mounts were prepared from E18.5 wild-type (P) and Ankrd6-/- (Q) embryos were also stained with an antibody against Lgn, a component of the apical compartmentation complexes. Lgn locates normally to the lateral side of the hair cell apical cortex in Ankrd6-/- embryos.

This figure was modified from figures published in Jones et al., 2014. Figure parts A-M were prepared by C. Jones.

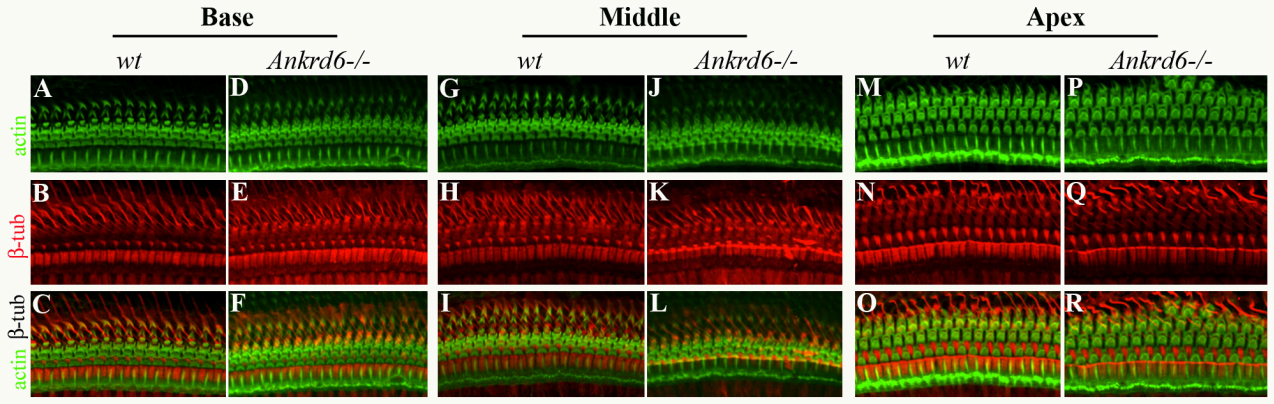

Fig. 4.3. Supporting cell PCP in the cochlea.

(A-R) Cochlear whole mounts were prepared from postnatal day 10 (P10) wild-type control (A-C, G-I, M-O) and Ankrd6-/- (D-F, J-L, P-R) animals, and stained for F-actin (green) and b-tubulin (red). Supporting cell polarity was shown by b-tubulin staining to visualize the orientation of their phallengeal processes.

This figure was published in Jones et al., 2014.

CHAPTER 5: Conclusions And Future Perspectives

The sensory hair cells of the organ of Corti are highly organized cells polarized along and apicobasal and longitudinal axis of the tissue. Over the past two decades the cellular and molecular processes crafting the precise structures in the hair cell have been studied and elucidated. A variety of proteins in the PCP pathway, blueprint proteins, regulators of actin and microtubules, and stereocilia adhesion proteins have been identified (see Chapter 1). The specific trafficking of these proteins to their final apically polarized domain is vital for the development, form, and function of the hair cell. When PCP proteins are not trafficked to the correct planar domain coordination of the direction of stereocilia and kinocilia orientation is lost (Curtin et al., 2003; Montcouquiol et al., 2006; Wang et al., 2006). Loss of expression of molecular blueprint proteins to the apical domain cause a loss of intrinsic polarity in the hair cell manifesting in dysmorphic, fragmented stereocilia (Tarchini et al., 2013). Additionally, in the gut epithelia, disruption of apical-basal polarity protein trafficking disrupts the apical microvilli (Feng et al., 2017; Knowles et al., 2015; Sobajima et al., 2014). This data indicates that trafficking is a vital part of the development of apical structures such as those seen in the organ of Corti hair cells.

Despite the necessity for precise trafficking of the proteins in these pathways it is not clear what regulates the trafficking of these proteins in the organ of Corti. Sensory hair cells are known to undergo endocytosis of their apical membranes at incredibly high rates and transcytose these membranes and proteins to the base and lateral surface of the cell (Griesinger et al., 2002, 2004). Rab11a, a component of the recycling endosome system responsible for ciliogenesis is uniquely positioned to sort

and transport apical proteins in the sensory hair cell. Rab11a was previously found to regulate proteins necessary for the apical localization of microvilli regulating proteins such as the ERM protein Ezrin in gut epithelia (Knowles et al., 2015; Sobajima et al., 2014).

In this dissertation, we used the organ of Corti to characterize the role of Rab11a in the development of the apical protrusions (Chapter 3) and the roles of Rab11a and Ankrd6 in the establishment of PCP (Chapters 3 and 4). By using conditional knockout of Rab11a in the organ of Corti we found that Rab11a regulates ciliogenesis through coordination with IFT88 and intrinsic polarity through stereocilia formation possibly through the regulation and trafficking of Usher proteins. Rab11a is vital for hearing and in adults is needed for stereocilia maintenance. Additionally, Rab11a affects the PCP pathway through a genetic interaction with Vangl2. These data clearly indicate that Rab11a is a component of the development of the apical structure of the sensory hair cell in the organ of Corti. Below I will discuss further details and future perspectives of these findings and present models of Rab11a's role in various aspects of hair cell development. Additionally, I will discuss the roles of Rab11a and Ankrd6 in PCP.

Rab11a alone is not responsible for global apical protein trafficking in the organ of Corti

Precise delivery of apical proteins is vital for the formation of apical structures in the organ of Croti. In epithelial cells in culture, Rab11 has been linked to this sorting activity at the trans Golgi network and through the recycling endosome through interactions with Rab11 family interacting proteins (Rab11-FIPs) and motor proteins as well as exocyst complex protein Sec15 (Casanova et al., 1999; Hales et al., 2002;

Horgan et al., 2010; Lapierre et al., 2001; Matsuzaki et al., 2011; Zhang et al., 2004). Additionally, studies in gut epithelia revealed that conditional knockout of Rab11a changed localization of microvilli associated proteins leading to malformed microvilli (Knowles et al., 2015; Sobajima et al., 2014). We used conditional knockout of Rab11a driven by Pax2Cre to deplete Rab11a from the sensory epithelia of the cochlea. We found that, despite deletion of Rab11a, a protein thought to be responsible for apical localization of many proteins in epithelial cells, in the organ of Corti PCP proteins Vangl2, Fz3, and LGN retained their apical localization (Figure 3.7). Additionally Spectrin, MyosinVIIa, and Radixin localization to the apical domain was not affected by loss of Rab11a (Figure 3.8). Likewise, basolateral localization of E-Cadherin was not altered by loss of Rab11a (Figure3.9).

We believe these results are due to a redundancy between Rab11a and Rab11b which share 91% protein identity. In fact, in the gut epithelia, Rab11b was found to compensate for the loss of Rab11a by increasing expression as visualized by staining with a Rab11b specific antibody (Knowles et al., 2015). The current understanding of Rab11 family proteins is muddled by studies that do not specifically disrupt only one of the Rab11 family proteins. Due to high sequence similarity, it is possible that knockout of Rab11a may also affect Rab11b or that expression of a dominant negative of one of these proteins also affects the other. To this end, it has been difficult to elucidate the specific roles of Rab11a and Rab11b in the cell. Studies have attempted to define roles for each protein and have concluded that Rab11a and Rab11b both regulate cellular trafficking through the recycling endosome but appear to discretely localize to distinct vesicles with differing cargos in many instances (Best et al., 2011; Butterworth et al.,

2012; Grimsey et al., 2016; Lapierre et al., 2003; Oehlke et al., 2011; Silvis et al., 2009). However, in some instances both Rab11a and Rab11b affect trafficking of the same proteins but to varying degrees or to different cellular destinations. For example, in HeLa cells trafficking of PAR1 is regulated by both Rab11a and Rab11b with Rab11a regulating trafficking to the lysosome for digestion and Rab11b regulating recycling back to the membrane (Grimsey et al., 2016). There are also cell type specific functions for Rab11a protein trafficking. For example Rab11a has been implicated in E-Cadherin trafficking in MDCK cells in culture but loss of Rab11a does not affect its localization in the gut epithelia or the organ of Corti (Desclozeaux et al., 2008; Knowles et al., 2015; Lock and Stow, 2005; Sobajima et al., 2014).

These data point to a model where Rab11a does not play a role in all apicobasal sorting mechanisms in the cell, but instead works with Rab11b and possibly Rab25 and other trafficking proteins to form discrete trafficking complexes with coat proteins and molecular motor proteins to help achieve precise trafficking regulation in a cell specific manner.

Regulation of kinocilia of the hair cell by Rab11a

Primary cilia are apical projections containing an axoneme surrounded by a membrane containing signaling molecules (Lim et al., 2011). Rab11 has been thoroughly studied in primary cilia formation *in vitro* finding that Rab11 is sorted into vesicles at the trans Golgi network with Rabin8 (Knodler et al., 2010; Wang et al., 2012). At the cilia, Rabin8 is activated by Rab11 that in turn activates Rab8 to allow for membrane extension and the formation of the cilia (Knodler et al., 2010; Nachury et al., 2007; Wang et al., 2012; Westlake et al., 2011). In order to investigate the specific role

of Rab11a in this process, we used Rab11a-null MEFs and assessed presence of ciliation upon serum starvation. We found that Rab11a specifically is necessary for cilia formation *in vitro* (Figure 3.4A-E). However, *in vivo* studies using Nestin driven Cre recombinase to create conditional Rab11a knockout did not show a ciliation defect in the brain (Sobajima et al., 2014). Likewise, loss of Rab11a alone did not cause kinocilia formation defects in the organ of Corti hair cells (Figure 3.4G,J-K).

Considering the vital role cilia play in development it is not unlikely that multiple redundant trafficking proteins are responsible for the localization of Rabin8 to the cilia and activation of Rabin8. These studies have not specified which Rab11 family member is responsible for trafficking or activation of Rabin8 at the cilia. We suggest that Rab11b could be compensating for Rab11a loss at least partially *in vivo*. To test this hypothesis, Rab11b conditional knockout mice are needed to create a compound Rab11a/Rab11b conditional knockout animal. These mice would not only benefit the understanding of cochlear biology, but also help elucidate the role of cilia formation in many other epithelia cell models. Additionally, use of a Rab11b specific antibody could determine if Rab11a loss increases Rab11b expression in this system.

Due to the strong evidence that Rab11a plays a critical role in cilia formation *in vitro*, we weakened the ciliation pathway in the cochlea to reveal a role for Rab11a in kinocilia formation *in vivo*. Through heterozygous conditional knockout of IFT88, a component of the intraflagellar transport system, in addition to loss of Rab11a, we revealed a significant loss of kinocilia in sensory hair cells in the inner hair cell row in addition to a slight but insignificant loss of kinocilia in the outer hair cell rows (Figure 3.4I-K). For the first time, we were able to show a function for Rab11a in ciliogenesis *in*

vivo. SEM analysis shed more light on this phenotype by revealing that kinocilia fail to form opposed to developing stunted axonemes (Figure 3.6). This suggests that there is a complete failure in kinocilia development in affected cells. However, since not all cells in the inner hair cell row are affected, perhaps a threshold of active Rab8 is needed with in the kinocilia in order to achieve proper cilia formation in the hair cell. I propose that wildtype and Rab11a knockout hair cells are able to shuttle enough active Rab8 to the forming kinocilia tip through compensating from other proteins in order to allow for membrane extension. However, with a partial loss of IFT machinery caused by heterozygous knockout of IFT88 there is sometimes too little Rab8 shuttled to the extending tip. Essentially, if a Rab8 threshold is not met, kinocilia formation cannot occur. In order to test this hypothesis, I could immunostain Rab11a knockout and Rab11a knockout with heterozyougs loss of IFT88 cochleas with Rab8 antibody to determine if there is a quantifiable change in Rab8 levels in the cell with or without kinocilia or between differing genotypes. This would help us understand why we see this phenotype and help elucidate differences seen in individual inner hair cells and perhaps differences seen between inner hair cells and outer hair cells.

We propose that the model for kinocilia formation is consistent with cilia formation described in the literature for epithelial cells *in vitro* (Figure 2.1). However, we have determined that Rab11a is not solely responsible for this process *in vivo* in the organ of Corti. We speculate that other Rab11 family members or trafficking proteins are compensating for Rab11a loss in our Rab11a knockout cochlea consistent with reports of Rab11b compensating for Rab11a in gut epithelial cells (Knowles et al., 2015). Our work and future work understanding the ciliation processes in the cochlea

and in other systems can help clarify the pathology or treatments for the variety of diseases that are caused by cilia defects termed “ciliopathies.”

Regulation of stereocilia formation in the hair cell by Rab11a

Rab11a has been clearly implicated in microvilli formation *in vivo* in gut epithelia where Rab11a loss leads to a reduction in length and width of microvilli and ectopic microvilli present in domains beyond the apical surface (Feng et al., 2017; Knowles et al., 2015; Sobajima et al., 2014). Stereocilia are modified microvilli structures on the apical surface of the hair cell with mechanotransduction ability that allows for auditory transduction. Like the gut epithelia, loss of Rab11a in the organ of Corti disrupts stereocilia formation although the phenotypes observed were inconsistent with the gut epithelia. Loss of Rab11a in hair cells leads to disruption in stereocilia patterning displaying missing, circular, flattened, or fragmented bundles more prevalent in the inner hair cell row than the outer hair cell rows (Figure 3.2). The microvilli of gut epithelia function to increase absorption of nutrients while the stereocilia of the cochlea respond to sound waves in the inner ear by deflecting to allow mechanotransduction. These divergent functions mean that the microvilli are fundamentally different and as such the differences in phenotype due to loss of Rab11a are not surprising. Nevertheless each system demonstrations a clear role for Rab11a in the formation of microvilli or microvilli derived structures.

The defects seen in gut epithelia due to loss of Rab11a have been explained by mislocalization of microvilli regulating proteins from the apical membrane (Knowles et al., 2015; Sobajima et al., 2014). Likewise, we suspected that similar proteins might be affecting stereocilia formation in the organ of Corti. We analyzed Spectrin, MyosinVIIa,

and Radixin localization in the hair cell to determine if Rab11a was affecting the localization of these proteins (Figure 3.8). Spectrin is a component of the cuticular plate necessary for the insertion of the stereocilia rootlet to anchor the stereocilia but was not affected by loss of Rab11a. Likewise MyosinVIIa, a motor protein necessary for stereocilia elongation was appropriately localized to the apical surface of hair cells when Rab11a was lost. Surprisingly, Radixin was also localized correctly to the apical membrane despite Ezrin, another ERM family member, being mislocalized in the gut epithelia (Dhekne et al., 2014; Knowles et al., 2015). The apical compartmentalization protein LGN thought to influence stereocilia shape retained apical localization with loss of Rab11a (Figure 3.7).

Because the stereocilia elongation proteins and stereocilia patterning proteins we tested did not reveal a mechanism for the disruption of stereocilia formation, we instead focused on a potential disruption of Usher proteins in the hair cell. Usher proteins include adhesion proteins, scaffold proteins, and motor proteins that allow for adhesion between stereocilia bundles and mechanotrasnduction. Similar to the phenotype in Rab11a conditional knockout cochlea, Usher protein mutations cause a fragmentation or splaying of stereocilia bundles thought to be due to the loss of adhesion between the stereocilia. Interestingly, the area adjacent to the stereocilia is known to undergo membrane recycling potentially including trafficking of adhesion complex proteins and Usher proteins Pcdh15 and VLGR1 undergo highly regulated variant specific trafficking in the hair cells (Griesinger et al., 2002, 2004; Zallocchi et al., 2012). Since loss of Rab11a in gut epithelia cause Mucin-like Protocadherin to lose its tight apical localization, and Rab11 regulates the localization of a variety of classical cadherins,

perhaps the localization of cadherins in the organ of Corti is also regulated by Rab11a (Knowles et al., 2015). We decided to investigate the hypothesis that Rab11a regulated trafficking of the adhesion molecules needed for these inter-stereocilia linkages.

Due to a lack of available Usher protein antibodies, we utilized LLC-PK1-CL4 cells, a kidney cell line with microvilli used to characterize Usher proteins localization and microvilli extension characteristics of Espin proteins (Zheng et al., 2014; Zheng et al., 2010). We used this model to determine if Rab11a affects the localization of the adhesion proteins in the stereocilia. Specifically, we found that expression of the Rab11a dominant negative construct caused a loss of specific apical localization of Cdh23 (Figure 3.12). As these cells have been used to model stereocilia in hair cells we speculate that Rab11a regulates the trafficking of Cdh23 and possibly other proteins necessary for stereocilia linkages in the sensory hair cells in the organ of Corti. Further analysis of these cells is needed to determine how reliably this phenotype occurs. I plan to quantify the localization change of Cdh23 by counting cells where Cdh23 is localized apically, basally, or both and comparing between WT and dominant negative Rab11a constructs. In order to further investigate this possibility, we could focus on the colocalization of Rab11a and Cdh23 during trafficking in these cells using confocal microscopy or live cell imaging. These constructs could also be used in biochemical analysis to determine specific interactions between the two proteins.

To further test this hypothesis we have collaborated to visualize inter-stereocilia links including the tip link responsible for mechanotransduction via scanning electron microscopy although these studies have not yet been completed. If these data reveal a loss of inter-stereocilia linkages it will further support our hypothesis that Rab11a is

responsible for Usher protein trafficking in the hair cell. In order to further understand if and how Rab11a affects interstereocilia links, we could utilize tip link mutants of Cdh23 and Pcdh15. It has been well established that homozygous mutations in Cdh23 or Pcdh15 cause a splaying of stereocilia similar to what we see with Rab11a loss in the cochlea while heterozygous mutation does not. By compounding either of these heterozygous mutants with Rab11a loss, we may be able to determine a genetic interaction between Rab11a and these adhesion proteins.

Through collaboration with Dongdong Ren, we observed auditory dysfunction in Rab11a conditional knockout mice through auditory brainsteam response (ABR) (Figure 3.11). Whole mount samples of these samples showed a distinct loss of stereocilia bundles in both inner and outer hair cells. We also observed disorganized stereocilia very apparent in the outer hair cell row. This suggests that Rab11a is needed for the maintenance of stereocilia as well as the formation of stereocilia.

Taking into consideration the data gathered in Chapter 3, we propose a model where Rab11a affects stereocilia formation and maintenance through the trafficking of adhesion molecules, likely Cdh23 and Pcdh15, to the stereocilia where they can form stereocilia linkages including tip links and transient lateral links. Loss of these adhesion molecules from the tip link would explain the degeneration of hair cells as hair cells that lose function often degenerate and the defects in ABR seen due to a loss of mechanotransduciton ability. Further research is needed to definitely conclude which trafficking pathways are being utilized by Usher to properly localize in the organ of Corti. However, we have a good model of Rab11a conditional knockout in order to further

study this process although further development of antibodies would be needed to answer these questions.

Further understanding of the planar cell polarity pathway from trafficking to downstream effectors

PCP has been studied in multiple systems in vertebrate and invertebrates. Although studies originated in *Drosophila* wing, recently the mammalian organ of Corti has become a well-established model for PCP due to the precise coordinated orientation of the kinocilia and stereocilia across the plane of the tissue. In Chapters 3 and 4 we investigated the roles that Rab11a and Ankrd6 in PCP. First I will discuss the potential role Rab11a is playing in PCP, then discuss how Ankrd6 helps complete the mammalian PCP pathway.

Rab11 has been implicated in trafficking of Vangl2, a core PCP protein *in vitro* and *in vivo* in *Xenopus* (Devenport et al., 2011; Kim et al., 2012; Ossipova et al., 2015; Ossipova et al., 2014). However, in mammalian systems Rab11 has not been directly indicated in trafficking of Vangl2. We determined that Rab11a is not necessary for asymmetric localization of core PCP proteins Vangl2 or Fz3 in the organ of Corti (Figure 3.7). Additionally, loss of Rab11a alone did not effect in the orientation of sensory hair cells (Figure 3.10). However, when the PCP system was weakened with by a heterozygous mutation in Vangl2, the outermost hair cells of the apex of the cochlea became significantly misoriented. We can conclude that this defect was not due to the loss asymmetrical localization of core PCP proteins but must be taking place downstream of the core PCP proteins.

The previously descried model could explain this defect in the PCP pathway where Rab11a is regulating the trafficking of stereocilia adhesion molecules such as Cdh23 and Pcdh15. Previous work by the lab has shown that mutations in Pcdh15 cause planar cell polarity defects (Chacon-Heszele et al., 2012). However there are some key differences observed between Pcdh15-3J heterozygous mutant and the Rab11a mutant including the Pcdh15 mutant displaying a phenotype in all hair cell types and displaying PCP defects in all hair cell rows (Chacon-Heszele et al., 2012). This could be explained by partial loss of Pcdh15 localization instead of complete ablation or functional deficiency in Pcdh15.

Alternatively, Rab11a could be responsible for trafficking of a downstream effector of the core PCP proteins that connects them to the stereocilia building proteins discussed in Chapter 1. This model would explain why we did not see a change in the stereocilia building and stabilizing proteins investigated in Figure 3.8. However, determining which downstream effector of the PCP pathway linking to stereocilia building would be a laborious process due to the many proteins identified in this process not including those that have yet to be identified. Since PCP regulates many aspects of development and organogenesis, our research could be applied to other systems leading to a further understanding of developmental processes.

In Chapter 4, we identified and characterized a homolog to the *Drosophila* PCP protein Diego, Ankrd6. Ankrd6 not only rescues loss of Diego in the fly wing and eye, but also partitions to a discrete apical domain of the organ of Corti cells and genetically interacts with core PCP protein Vangl2 (Figures 4.1 & 4.2) (Jones et al., 2014). Previously, core PCP proteins from the fly PCP pathway had been identified, as had the

PCP associated proteins except the diego homolog. This discovery helped complete the mammalian PCP protein pathway further suggesting the fundamental similarities between the fly and mammalian PCP signaling pathways (Figure 1.3).

Summary

The structure of the mammalian organ of Corti hair cells is precisely developed to meet its auditory function. The molecular pathways underlying hair cell development must be tightly regulated in order to achieve the hair cell's final form. Although these molecular pathways have been investigated, the trafficking events that allow the proteins in the pathways to end up in their final destinations have yet to be thoroughly explored. To this end, this dissertation investigated the role of Rab11a, a trafficking protein implicated in the recycling endosome, ciliation, and microvilli formation in epithelial cells. In this dissertation, I present evidence that Rab11a specifically regulates cilia formation in cultured cells and in kinocilia development in the organ of Corti, regulates stereocilia formation, and plays a role in PCP signaling downstream of core PCP proteins (summarized in Figure 5.1). I proposed a model where Rab11a affects kinocilia formation through the model developed in the Rab11 ciliation field (Figure 2.1). I proposed that Rab11a affects stereocilia development through trafficking of adhesion proteins such as Cdh23 and Pcdh15 to the stereocilia explaining the fragmented phenotype seen in Rab11a null cochlea. This model would also explain the mild PCP phenotype seen in the Rab11a null cochlea weakened with a heterozygous Vangl2-looptail mutation as Pcdh15 mutant cochlea show a PCP defect. This dissertation also analyzed the function of Ankrd6 in the mammalian cochlea as a downstream effector of core PCP proteins and a homolog of the *Drosophila* PCP protein

Diego. This dissertation provides insight into the importance of trafficking to sensory hair cells in the organ of Corti provides additional *in vivo* data for the role of Rab11a in ciliogenesis, microvilli development, and cellular trafficking.

Figures

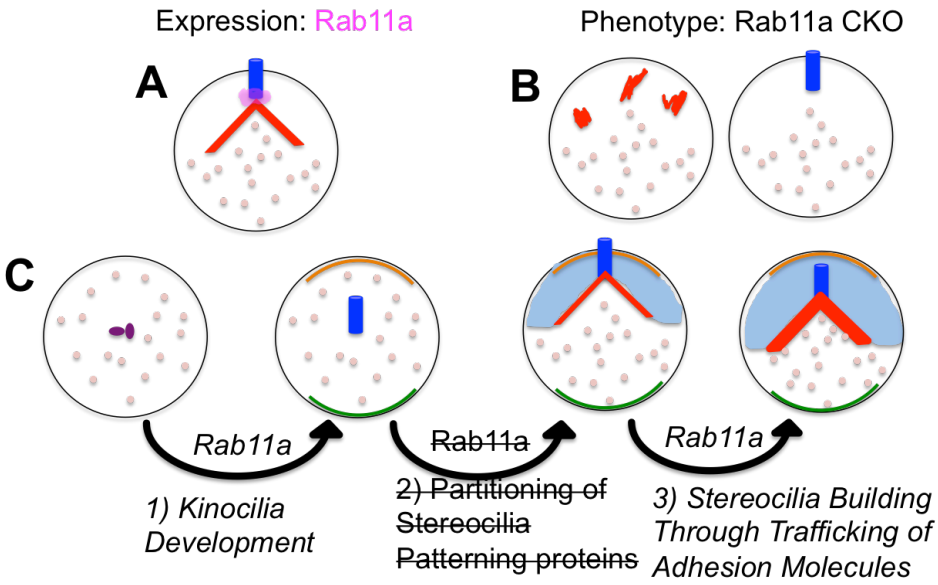

Figure 5.1. Summary and Hypothesis Model for Rab11a in the Cochlear Hair Cells

(A) Rab11a is expressed adjacent to the basal bodies in the hair cell at the base of the kinocilia.

(B) Phenotypes of Rab11a conditional knockout in the organ of Corti include a loss of intrinsic polarity manifesting in the absence of kinocilia and fragmented, malformed, or missing stereocilia.

(C) (1) Rab11a regulates ciliogenesis of the transient kinocilia in the sensory hair cells likely through the model explained in figure 2.1. **(2)** Despite a genetic interaction with Vangl2 causing PCP defects in the cochlea, Rab11a is necessary for the partitioning of PCP proteins or LGN. The phenotype may be due to trafficking of downstream effectors of PCP. **(3)** Fragmentation of the stereocilia in conditional knockout cochlea can be explained by a loss of adhesion molecules such as Cdh23 and Pcdh15 to the stereocilia. Additionally loss of these molecules cause PCP defects discussed in (2).

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