



Development and Patterning of the Cochlea: From Convergent Extension to Planar Polarity

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Within the mammalian cochlea, sensory hair cells and supporting cells are aligned in curvilinear rows that extend along the length of the tonotopic axis. In addition, all of the cells within the epithelium are uniformly polarized across the orthogonal neural–abneural axis. Finally, each hair cell is intrinsically polarized as revealed by the presence of an asymmetrically shaped and apically localized stereociliary bundle. It has been known for some time that many of the developmental processes that regulate these patterning events are mediated, to some extent, by the core planar cell polarity (PCP) pathway. This article will review more recent work demonstrating how components of the PCP pathway interact with cytoskeletal motor proteins to regulate cochlear outgrowth. Finally, a signaling pathway originally identified for its role in asymmetric cell divisions has recently been shown to mediate several aspects of intrinsic hair cell polarity, including kinocilia migration, bundle shape, and elongation.

The mammalian cochlea is a remarkable example of cellular engineering. Selective pressures related to the ability to perceive and discriminate a wide array of auditory frequencies have resulted in the formation of a complex structure with examples of cellular precision at multiple levels (Manley 2017). At a gross level, the cochlea can be modeled as a logarithmic spiral (see britannica.com/science/logarithmic-spiral) that is generally conserved in terms of length within individuals of the same species. But the degree of organizational precision is even more striking at the tissue and cellular levels. Extending from the base to the apex of the spiral, the organ of Corti (OC) comprises highly ordered rows of hair cells (HCs) interdigitated

with similarly ordered rows of supporting cells (SCs) (Kelley et al. 2009; Groves and Fekete 2012). The number and arrangement of each cell type is nearly invariant along a given OC or between OCs in disparate mammalian species. Similarly, each HC is topped with an elegant stereociliary bundle that has a V shape. The vertices of all HCs are uniformly aligned to point toward the outer (distal/lateral/abneural) edge of the spiral. The alignment of these bundles is a wonderful example of form following function as each bundle is directionally sensitive and can only respond to deflections along the neural–abneural axis. Understanding the cellular and molecular processes that must work in concert to achieve this level of organization has long

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been a goal of developmental and auditory biologists. Although a complete picture of how this structure forms is still emerging, our understanding of several aspects of this process has increased significantly through the use of mouse genetics and advanced microscopy. This article will review our existing understanding of several crucial processes that occur during cochlear formation and patterning and will discuss how those processes meld together to achieve such a high degree of developmental precision.

INITIAL OUTGROWTH OF THE COCHLEAR DUCT

Soon after the closure of the otic pit to form the otocyst, two outpocketings begin to extend from the dorsal and ventral poles of the otocyst (Wu and Kelley 2012). The dorsal extension will form the relatively straight endolymphatic duct, whereas the ventral extension will coil to give rise to the cochlear duct. Different regions of the duct will ultimately give rise to the three walls of the scala media: Reissner's membrane, the lateral wall including the stria vascularis and spiral ligament, and the basilar membrane comprised of the OC and inner and outer sulci. Counter gradients of Wnt and Hedgehog originating from the dorsal and ventral regions of the neural tube, respectively, play key roles in the determination of dorsal (vestibular) and ventral (auditory) identity across the axis of the otocyst (Riccomagno et al. 2002; Bok et al. 2007; Brown and Epstein 2011), but how ventral identity mediates the formation, extension, and coiling of the cochlear duct is unknown.

Lineage-tracing studies using *Sox2^{creErt2}* to drive expression of green fluorescent protein (GFP) indicate that the cells that initially comprise the extending duct are derived from progenitor cells located in the ventral region of the otocyst at the outset of extension (Gu et al. 2016). Moreover, the spatial distribution of different, probably lineage-restricted progenitors suggests that the general spatial pattern of cell types is established at the outset of extension. Although the specific signaling pathways that mediate overall growth of the duct are unknown, most of the cell types within the duct continue to pro-

liferate through at least the early postnatal time period in the mouse (Ruben 1967; Chen and Segil 1999), suggesting that increasing cell numbers probably play a role in driving outgrowth.

In contrast with all other otic progenitor cells, the prosensory cells that give rise to the HCs and SCs that form the OC, become postmitotic by E14, at least 7 days prior to end of active outgrowth (Ruben 1967; Chen and Segil 1999; Lee et al. 2006). At this stage, the length of the duct is generally less than half of its final length, suggesting that in the mouse some prosensory cells will need to move a distance of $\sim 3000 \mu$ to reach their final position at the extreme cochlear apex (McKenzie et al. 2004; Yamamoto et al. 2009; Driver et al. 2017). Initial observations of the changes in the distribution of progenitors that occur during cochlear outgrowth led to the suggestion that a conserved morphogenetic process termed convergence and extension (CE) plays a role in prosensory outgrowth (Chen et al. 2002). In other systems, CE is characterized by the active migration of cells located at the periphery of an epithelial field toward the midline (Keller et al. 2000; Skoglund and Keller 2010; Solnica-Krezel and Sepich 2012). As cells at the periphery move inward, they intercalate between existing cells. This creates extension orthogonal to the direction of inward movement. Although initial observations were consistent with a CE-mediated process, subsequent morphological analyses and time-lapse imaging indicated that prosensory extension also occurs through radial intercalation and cellular growth (Driver et al. 2017). At E14, the prosensory cell population is arranged in a highly pseudostratified epithelium with an average of 10 cells stacked between the luminal surface and the basement membrane, with every cell within that stack extending projections that contact both the luminal and basal surfaces (Fig. 1). Between E14 and E16, the cells undergo radial intercalation causing the stratification of the epithelium to decrease to approximately five cells, whereas the height of each cell decreases. This rearrangement has the potential to generate a force oriented orthogonal to the direction of intercalation with one possible outcome of this force generation being displacement of prosensory cells toward the apex of the

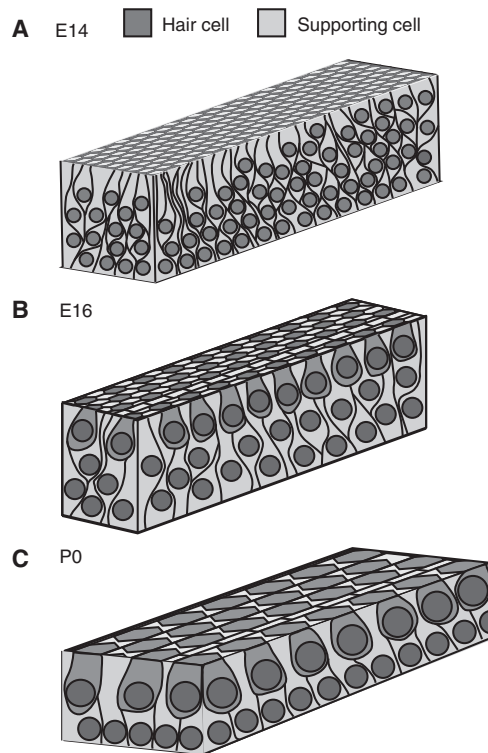


Figure 1. Changes in distribution of prosensory cells during cochlear outgrowth. Each drawing represents a region near the midbase of the cochlea. (A) At E14, the epithelium is highly pseudostratified. There is no cellular organization and no morphological differentiation has occurred. (B) At E16, immature hair cells have begun to become organized into rows and stratification has decreased although more than two layers of cells are still present. Note the overall decrease in cell density as a result of cellular migration and extension. (C) By P0, the mature cellular pattern is present with a luminal layer of hair cells resting on a basal layer of supporting cells.

cochlea. Somewhat surprisingly, the width of the prosensory domain along the neural–abneural axis is unchanged over the same time period, a result that is inconsistent with ongoing convergence. However, there is a concomitant average increase in individual cell volume of ~66% ($\pm 30\%$) depending on cell type (HC vs. SC), which masks some ongoing convergence (see below). Between E16 and P0, cellular migration and intercalation decrease but cellular growth (measured as an increase in volume), in particular of the HCs, continues. These results suggest

that at early time points, outgrowth is driven by a combination of cellular intercalation, convergence, and increasing cellular size, although at later time points an increase in cell size seems to be the primary driver of extension.

The core planar cell polarity (PCP) signaling pathway has been shown to regulate many processes that involve collective migration and cellular extension including neural tube closure, gastrulation, and epiboly (Goodrich 2008; Simons and Mlodzik 2008; Carreira-Barbosa et al. 2009; Skoglund and Keller 2010). Consistent with these observations, shortened cochleae have been reported for mouse lines carrying mutations in different members of the PCP pathway. In particular, mutations in *Vangl2*, *Dsh1 + 2*, *Wnt5a*, *Dchs1*, and *Fat1/4* result in shorter cochleae than those in controls (Montcouquiol et al. 2003; Wang et al. 2006a,b; Qian et al. 2007; Mao et al. 2011; Saburi et al. 2012). However, other PCP mutants, such as *Frz3/6* and *Celsr1*, which play a role in stereociliary bundle orientation (see below), were not reported to have short cochleae (Curtin et al. 2003; Wang et al. 2006b). It is not clear whether this is a result of functional redundancy, genetic compensation, an absence of a role in cochlear outgrowth, or simply restricted expression of these proteins only in HCs. Most of the PCP mutants with shortened cochleae also contain cellular patterning defects in the distal end, suggesting that outgrowth and cellular patterning are linked to some extent (see below). For many of the mutants described above, phenotypes related to both cochlear outgrowth and polarization are exacerbated on a *Vangl2^{Lp/+}* background demonstrating genetic and potential biological interactions between many of these factors and *Vangl2*, although some of these effects might be caused by the dominant-negative effect of the *Vangl2^{Lp}* allele (Yin et al. 2012).

Examination of mutations affecting molecules that directly mediate changes in the cellular cytoskeleton and that may be modulated directly or indirectly through the PCP pathway have shown more extensive effects on cochlear extension. For instance, targeted deletions of the GTPase *Rac1*, the microtubule motor *Kif3a*, or IFTs *Ift88*, *Ift20*, or *Ift25* or disruption of

the nonmuscle Myosin II motor complex lead to variable shortening of the duct (~50%) and concomitant defects in patterning that are most commonly manifested as supernumerary rows of outer (OHCs) and inner hair cells (IHCs) located at the apex (Grimsley-Myers et al. 2009; Yamamoto et al. 2009; Sipe and Lu 2011; May-Simera et al. 2015). Inhibition of Myosin II either in vitro using Blebbistatin or in vivo through expression of a dominant-negative form of *Myh10* leads to significant changes in several aspects of prosensory cell morphology including cell size and the number of four-cell vertices, a marker of ongoing cellular intercalation (Driver et al. 2017). Briefly, during active cellular intercalation, the luminal patterning of cells changes. A common indicator is the formation junctions that contain more than three cells. Therefore, mapping four-cell vertices in fixed tissue can be used as a proxy for cellular intercalation events. At a subcellular level, work from Lu and colleagues has shown that PTK7, a receptor tyrosine kinase-like molecule that was initially identified as a noncanonical PCP protein, facilitates junctional localization of Myh10 (a Myosin II heavy chain) in developing SCs (Lee et al. 2012). This localization provides a scaffold for Myosin II-mediated contractile forces, which contract the luminal surfaces of SCs at late embryonic stages (Ebrahim et al. 2013). Consistent with this conclusion, cochlea from mice expressing a dominant-negative *Myh10* construct throughout cochlear development have SCs that show defects in luminal contraction, a decrease in the total area at the cells' luminal surface (Yamamoto et al. 2009). However, the effects of Myosin II are not limited to luminal contraction as *Myh10^{DN}* cochleae also contain supernumerary rows of misshaped pillar cells. Finally, expression of *Myh10^{DN}* only in HCs results in smaller HCs and a shortened cochlea even though overall cellular patterning is normal (EC Driver and MW Kelley, unpubl.).

In contrast with the reverse genetic results described above, forward genetic analysis also has the potential to provide insights regarding cochlear outgrowth. In particular, a collection of human disorders referred to as cochlear hypoplasias and/or Mondini disorders (depending on

severity) typically include a shortened cochlea and some degree of low-frequency hearing loss (Jackler et al. 1987; Sennaroglu and Saatci 2002). Subsequent analyses have examined cochlear structure, largely reporting a relatively normal OC and duct in basal regions but cystic or malformed structures toward the apex. Only a few of the causative genes for cochlear hypoplasia or a Mondini disorder have been identified. The anion transporter, *SLC26A4*, also known as Pendrin, underlies ~50% of all cases of Pendred syndrome, which typically features a Mondini dysplasia (Pourova et al. 2010; Huang et al. 2011). In these individuals, the cochlea is typically short, swollen, and distended, as are other regions of the inner ear, suggesting that the defect in cochlear outgrowth may be a result of more global defects in inner ear formation. Other genes that have been linked with shortened cochleae in humans include *KCNJ10*, *FOXI1*, *POU3F4*, and *FGF3* (Jonard et al. 2010; Pique et al. 2014). However, as is the case for *SLC26A4*, the broad expression of these genes throughout the developing otocyst or periotic mesenchyme prevents inferences regarding the specific roles of these genes in cochlear outgrowth. Similarly, although rare, there are genetic mutations resulting in congenital low-frequency sensorineural hearing loss (cLFSNHL) that have been mapped to *WFS1*, *DIAPH1*, and *MYO7A* (Lynch et al. 1997; Bessalova et al. 2001; Cryns et al. 2003; Street et al. 2004). Although shortened cochleae have not been reported for any patients with cLFSNHL, a potential role for the mouse ortholog Diaphanous1 in outgrowth seems possible considering its known interactions with GTPases and the cytoskeleton (Purvanov et al. 2014; Wu et al. 2015).

Finally, some patients with Pallister-Hall syndrome (PHS), which is caused by a truncating mutation in the Sonic hedgehog target gene *GLI3*, resulting in a partial loss of hedgehog function, have been shown to suffer from LFSNHL, and magnetic resonance imaging (MRI) analysis of the temporal bones from one patient indicate cochlear hypoplasia consistent with shortening (Driver et al. 2008; Avula et al. 2012). Analysis of *Gli3^{Δ699/Δ699}* mice, a model for PHS, similarly indicated a shortened cochlear duct and cochlear outgrowth assays showed de-

fects in extension in response to inhibition of Sonic hedgehog signaling (EC Driver and MW Kelley, unpubl.). Overall, these results provide strong evidence for a role for hedgehog signaling in cochlear outgrowth. Specific deletion of *Shh* in the developing spiral ganglion results in a similar degree of shortening to that observed in PHS (Bok et al. 2013), suggesting that Shh originating in the spiral ganglion may act to stimulate some aspect(s) of cochlear extension although the specific roles of Shh have not been determined yet.

COCHLEAR MOSAIC

During the course of cochlear development and outgrowth, HCs and SCs become arranged in a precise mosaic. A key initial step in this process is the sorting of progenitor cells into HCs and SCs, which occurs through activation of the notch signaling pathway (Lanford et al. 1999, 2000; Kiernan et al. 2005; Brooker et al. 2006). But, analysis of cochleae from notch mutants indicates that although there is a disruption in the number of progenitors that become committed to develop as HCs, an alternating mosaic of HCs and SCs still forms (see Fig. 2) most likely as a result of the recruitment of additional SCs by developing HCs (Woods et al. 2004; Kiernan et al. 2005; Hartman et al. 2010; Pan et al. 2013). It is important to consider that notch signaling occurs concomitantly with active outgrowth. Therefore, interactions between progenitor cells, and activation of the notch pathway, can change in response to changes in cell–cell contacts. Consistent with this hypothesis is the demonstration that inhibition of cochlear outgrowth alters cell fates causing fewer cells to develop as HCs (Driver et al. 2013), suggesting that progenitor cells may use active migration as a mechanism to escape notch activation. However, this observation also suggests that cellular rearrangement occurs subsequent to determination of cell fate, raising questions regarding how HCs and SCs pattern into such a precise mosaic.

Two evolutionarily conserved transmembrane immunoglobulin-like molecules called Nectins participate in the generation of the mosaic pattern of the OC. Nectin 1 and 3 are differentially expressed on HCs and SCs, respectively,

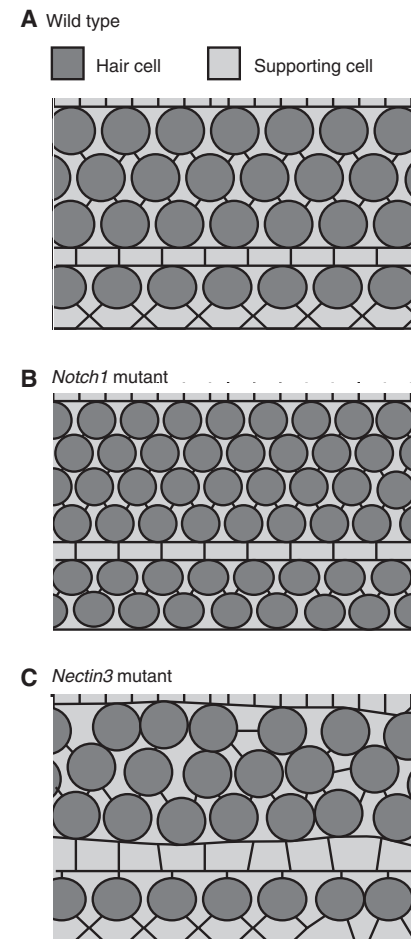


Figure 2. Effects of different mutations on cell patterning. (A) Surface view of the wild-type organ of Corti (OC), illustrating the normal pattern of hair cells and supporting cells. (B) Disruption of notch signaling, as occurs following deletion of *Notch1*, leads to an overproduction of hair cells but the mosaic of hair cells and supporting cells is still maintained, most likely as a result of recruitment of supporting cells by hair cells. (C) In contrast, the number of hair cells and supporting cells in the OC from a *Nectin3* mutant does not significantly differ from wild type, but hair cells are observed to be in contact, indicating a disruption in cellular patterning.

and can bind via both homo- and heterophilic interactions (Togashi 2016). Deletion of either *Nectin1* or *Nectin3* leads to defects in the cochlear mosaic, most commonly HC–HC contacts (Fig. 2). Although the changes in cochlear patterning in these mutant lines are largely con-



sistent with a preferential heterophilic binding between Nectin1/HC and Nectin3/SC, there are several factors to consider. In particular, the patterning defects in *Nectin1* mutants are relatively mild by comparison with the more severe phenotype observed in *Nectin3* mutants. In addition, analysis of Nectin1 expression in *Nectin3* mutants indicates a nearly complete absence of Nectin1, suggesting either an interdependence between cells or the possibility that Nectin3 is expressed in both SCs and HCs, or a non-cell-autonomous role of Nectin3. Therefore, generating cell-type-specific deletions for both *Nectins* in HCs and/or SCs is needed to fully understand the molecular role of each protein. But, the phenotypes observed in the cochleae of these animals strongly suggest that heterophilic interactions between developing HCs and SCs are critical, consistent with these interactions being stronger than homophilic ones (Togashi et al. 2011).

PLANAR CELL POLARITY AND ORIENTATION OF STEREOCILINARY BUNDLES

As discussed, cochlear HCs serve as a unique model for the study of cellular polarity. In the early stages of differentiation, the apical region of the HC undergoes a complex morphogenic process to form and orient the mechanosensory stereociliary bundle. Each stereociliary bundle is derived from the single true cilium that exists on all epithelial cells and a cluster of microvilli that adorn the apical surface of the cell. As bundle development begins, the axoneme of the primary cilium (future kinocilium), which initially projects from the center of the apical surface of the young HC, grows in size and migrates eccentrically toward the abneural edge of each HC (Mbiene et al. 1984; Lim and Anniko 1985; Forge et al. 1997; Lepelletier et al. 2013). By P15, the cilium and microvilli have remodeled to form a cohesive U-, V-, or W-shaped stereociliary bundle composed of tens to hundreds of actin-rich stereocilia arranged in precise rows to create a staircase pattern. At the vertex of each stereociliary bundle is a kinocilium, which serves as the “guidepost” for hair bundle planar orientation. The kinocilium is anchored by a

basal body located at its base and positioned just below the apical membrane in an abneural notch in the cuticular plate called the fonticulus.

INITIAL DISCOVERY OF THE ROLE OF PCP GENES IN STEREOCILINARY BUNDLE ORIENTATION

A crucial role for the core PCP pathway in the orientation of stereociliary bundles was initially described in 2003, when the analysis of the cochleae of a spontaneous mutant mouse line (looptail) bearing a mutation in *Vangl2*, a vertebrate homolog of the *Drosophila* PCP gene *vang*, revealed formed but misoriented, stereociliary bundles (Fig. 3A) (Montcouquiol et al. 2003). Subsequent studies identified similar roles for other vertebrate homologs of *Drosophila* core PCP genes, including *Celsr1*, *Dishevelled*, and *Frizzled* (Curtin et al. 2003; Wang et al. 2005, 2006b). Further studies indicated that the establishment of PCP in the cochlear and vestibular epithelia depended on the planar asymmetric localization of core PCP proteins to specific sides of HC and SC (Fig. 3B), similar to the process that occurs in *Drosophila* (Adler 2012).

Vangl2 and *Prickle 2* localize at boundaries between the neural sides of HCs and the abneural sides of SCs, and opposite to *Frizzled 3* and *6*, whereas *Dishevelled 2* and *3* localize at the distal side of the HC between the abneural sides of HCs and the neural sides of SCs (Montcouquiol et al. 2006; Wang et al. 2006b; Deans et al. 2007; Yin et al. 2012; Ezan and Montcouquiol 2013). More detailed examinations of the phenotypes in specific core PCP mutants have shown differential sensitivity based on HC type or position. For instance, although third-row OHCs are most affected in *Vangl2* mutants, in *Fz3/6* double mutants IHCs show the most severe degree of misorientation (Fig. 3A,C) (Montcouquiol et al. 2003; Wang et al. 2006b; Yin et al. 2012). But, although bundles are misoriented in core PCP mutants, the bundles themselves still develop with a characteristic polarity, as illustrated by the formation of a “V” or “W” shape, and the kinocilium still migrates to the cell cortex, indicating that the intracellular polarity is not disrupted. This phenotype contrasts with other

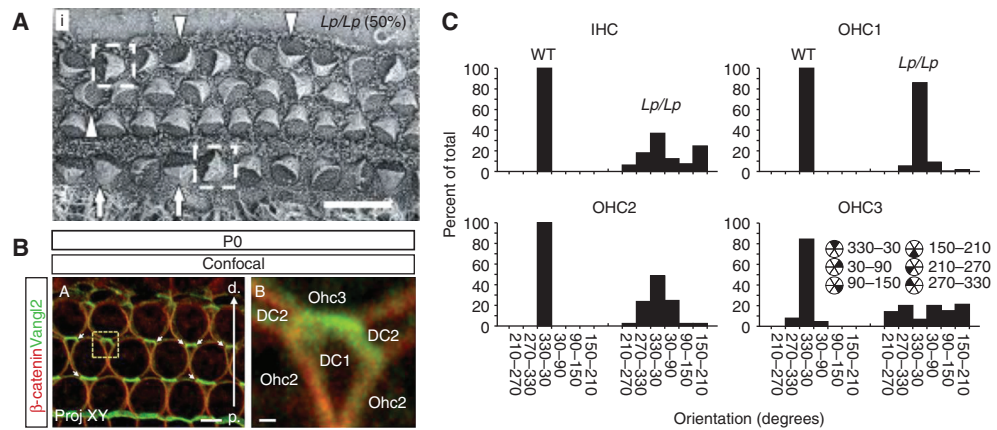


Figure 3. Vangl2 regulates stereociliary bundle orientation in the cochlea. (A) Scanning electron micrograph of the organ of Corti from a *Vangl2*^{Lp/Lp} mouse at E18.5. Stereociliary bundles show varying degrees of misorientation (indicated by arrowheads, arrows, and boxed cells). (B) Confocal image of the surface of the outer hair cell (OHC) region from the organ of Corti of a P0 mouse. Vangl2 is localized to the junctions between the distal sides of Deiters cells (DCs) and the proximal sides of OHCs. (C) Frequency histograms illustrating the distribution of bundle orientations, based on cell type, in control and *Vangl2*^{Lp/Lp} cochleae at E18.5. Note that although bundles in wild-type (WT) animals show a limited distribution centered around the optimal (0°) orientation, in *Vangl2*^{Lp/Lp} bundles show varying degrees of misorientation based on location. Scale bars, 10 μm (A); 4 μm (B, left); 0.5 μm (B, right). IHC, Inner hair cell. (Panels A and C from Montcouquiol et al. 2003; reproduced, with permission, from *Springer Nature*. Panel B from Giese et al. 2012; reproduced, with permission, from the authors.)

mutations that may lead to changes in both bundle orientation and shape. These include the genes within the Ush1 complex (Lefevre et al. 2008) as well as genes that underlie ciliopathic diseases such as Bardet–Biedl syndrome (BBS), Meckel–Gruber syndrome (MKS), and Alström syndrome (Alms) (May-Simera 2016). All of these disorders include hearing loss and therefore reflect the functional role of orientation. However, because bundle shape and/or mechanotransduction are also altered with these syndromes, it is not possible to determine how much of the auditory pathology should be attributed to defects in orientation. More recently, the mechanisms that mediate intrinsic cellular polarity have identified and will be discussed below.

GPSM2-LGN AND PAR COMPLEXES DETERMINE KINOCILIUM MOVEMENT AND HAIR BUNDLE SHAPE

As discussed, the first indication of bundle polarization is the centrifugal migration of the developing kinocilium toward the abneural edge of each HC. This process begins around E15, well

after the initial commitment of HCs, which occurs about 2 days earlier (E13) based on expression of the HC markers *Atoh1* and *Myosin 6* (Chen et al. 2002; Montcouquiol et al. 2003). An aster of microtubules originating from the base of the kinocilium (the basal body) and extending beneath and parallel to the apical surface of each HC was described in newborn animals (Hallworth et al. 2000). However, this microtubule aster has also been observed as early as E13.5 (M Montcouquiol, unpubl.), at which time the primary cilium/immature kinocilium has not yet migrated to the periphery of the cell. This suggests the existence of a microtubule-organizing center (MTOC) (Hallworth et al. 2000) that could guide the direction of basal body and kinocilial migration. In 2013, the similarity between these structures and microtubule-based asters that form during mitosis led to an examination of the role of the spindle-related heterotrimeric G protein α -subunit/ $G_{\alpha 13}$ in bundle development and orientation (Ezan and Montcouquiol 2013; Ezan et al. 2013; Tarchini et al. 2013).

Ezan et al. (2013) observed an abneural crescent of $G_{\alpha 13}$ in IHCs in the apical turn of E15.5

rat cochleae (the approximate equivalent to an E13.5/E14 in a mouse) and in OHCs a day later. Initially, $G_{\alpha i3}$ protein covers the entire apical surface of the HCs, within which there is a central cilium. The crescent of $G_{\alpha i3}$ is then formed, before the migration of the cilium, apparently playing an instructive role in the subsequent migration of the kinocilium (Fig. 4A,B). A similar dynamic profile was reported shortly after for the G-protein-signaling modulator 2 (Gpsm2; also known as LGN in mammals or Pins in invertebrates), a well-known and conserved partner of $G_{\alpha i}$, and for Inscuteable (Insc), a binding

partner of Pins (Tarchini et al. 2013). The same two studies revealed that there was, on the opposite side of the cell, a neural complex composed of the atypical protein kinase C (aPKC) protein and one of the members of the partitioning-defective (Par) family, Par6b. This complex abuts the shortest stereocilia row of the bundle. Therefore, Gpsm2-LGN/ $G_{\alpha i3}$ /Insc and aPKC/Par6b effectively establish asymmetric boundaries along the apical surface of each developing HC to delineate the shape of the hair bundle. In at least one study, both complexes were also shown to be asymmetrically enriched at cellular

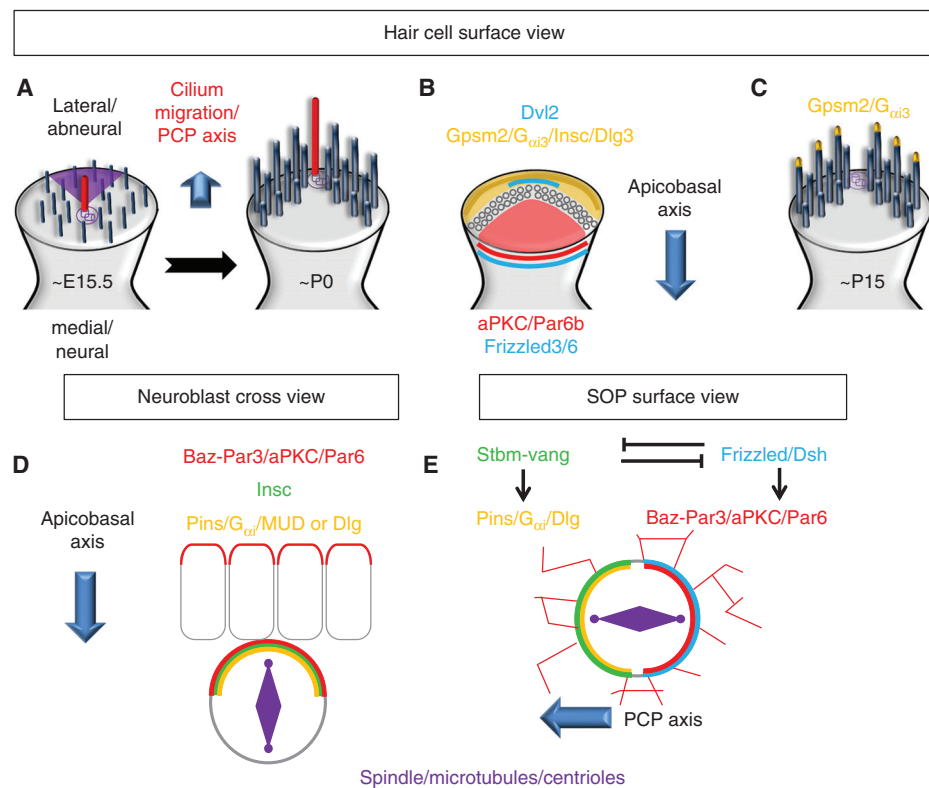


Figure 4. Differences in Gpsm2–Pins/ $G_{\alpha i}$ localization between hair cells, *Drosophila* neuroblasts, sensory organ precursor (SOP). (A,B) In hair cells, between E15.5 to P0, the migration of the kinocilium toward the lateral/abneural side is instructed by an apical accumulation of Gpsm2/ $G_{\alpha i3}$ /Insc/Dlg, whereas an aPKC/Par6 complex is restricted to the medial/neural side. Of note, Daple expression is restricted to the apicolateral junction (darker orange/brown). (C) In later stages, Gpsm2/ $G_{\alpha i3}$ accumulates at the tip of the longest stereocilia. (D) In delaminating neuroblasts, the Par-6/Baz-Par-3/aPKC and the Pins/ $G_{\alpha i}$ /Mud or Dlg complexes colocalize at the apical cortex and control the apicobasal orientation of the spindle. Pins/ $G_{\alpha i}$ /Mud or Dlg are recruited to the apical cortex through the presence of Insc. (E) In SOPs, Strabismus (Stbm)-Vang, localizes to the anterior cortex and recruits Pins, where they both work to restrict the Par complex to the posterior cortex, maintaining the spindle orientation parallel to the surface of the epithelium. PCP, planar cell polarity.

tight junctions (TJs) located just below and lateral to the apical membrane (Ezan et al. 2013). Inhibition of Gpsm-LGN or $G_{\alpha i}$ through either genetic mutations or exposure to PTX resulted in three major phenotypes (with varying degrees of severity): (1) misorientation of the hair bundle (similar to a classic PCP phenotype); (2) disruption of the centrifugal migration of the kinocilium (in extreme cases the kinocilium remained in the center of the cell); and (3) abnormally shaped (mostly flat or split) stereociliary bundles. The results of these two studies support a model in which the molecular machinery required to direct mitotic spindle orientation during asymmetric cell division is also adapted to control kinocilium migration and hair bundle shape and orientation (see below).

An intriguing result from the studies described above was the observation that Gpsm2-LGN/ $G_{\alpha i}$ complexes were localized to two different regions of each HC, the apical crescent and the TJ, suggesting the possibility that these two pools serve different functions. Access to different protein partners could lead to activation of different signaling pathways with unique functional consequences. For example, the apical proteins could limit the neural and abneural expansion of the stereociliary bundle and determine its final shape, whereas the TJ-localized complex could control the anchoring of and the pulling forces on the microtubule aster and, eventually, the final position of the kinocilium (see below). However, because these processes occur in the same cell and with limited temporal separation, it may be very difficult to dissociate the two effects. Although the factors that direct/limit the distribution of Gpsm2-LGN/ $G_{\alpha i}$ complexes have not been determined, deletion of *Insc* leads to a marked decrease in the accumulation of Gpsm2-LGN and $G_{\alpha i}$ in the apical crescent, suggesting a role for *Insc* in this process (Tarchini et al. 2016).

CANONICAL AND NONCANONICAL PATHWAYS

It is important to consider that noncanonical interactions of G proteins that involve direct interactions with cytoskeletal elements bypass

classical signaling pathways (Ahmed and Angers 2013). One example of such a noncanonical pathway is the role of $G_{\alpha i}$ in the regulation of the mitotic spindle during cell division (Cho and Kehrl 2008; Gönczy 2008). In this case, the GDP-bound form of G_{α} rather than the GTP-bound is the important form in force generation. In contrast with canonical G_{α} -signaling, interaction with a 7-TM receptor is replaced by an intracellular guanine nucleotide exchange factor (GEF) such as Ric8 that activates $G_{\alpha i/o}$ (Tall and Gilman 2005; Wilkie and Kinch 2005). Importantly, pertussis toxin blocks the binding of Ric8A to $G_{\alpha i}$, preventing its GEF activity for $G_{\alpha i}$ (Woodard et al. 2010). Thus, PTX does not simply prevent the G_{α} proteins from interacting with their cognate 7-TM receptors but also blocks the binding of Ric-8A to $G_{\alpha i}$, thus preventing its GEF activity for $G_{\alpha i}$.

Recently, deletion of *Daple*, another GEF, was shown to cause significant disruptions in kinocilium movement and hair bundle orientation and shape (Siletti et al. 2017). *Daple* was identified as a Dvl-binding protein and is asymmetrically located on the lateral/abneural side of HCs with Dvl2 (although possibly not in overlapping domains) but was not detected at the apical membrane crescent. The investigators suggested that the protein could be the link between core PCP and Gpsm2/ $G_{\alpha i}$ signaling in the cochlear epithelium. Although this hypothesis is appealing, many questions remain to be addressed. For instance, Aznar and colleagues (2018) showed that *Daple* and the PDZ-binding motif (PDZ-BM) of Fz compete for binding to the PDZ-domain of Dvl, whereas Dvl and $G_{\alpha i}$ compete for two distinct modules on *Daple*. The formation of a Fz/Dvl/*Daple* complex leads to the generation of a GTP-bound form of G_{α} , the “classical” activated form of $G_{\alpha i}$. This would suggest that *Daple* (maybe with Ric8) counteracts the function of Gpsm2/ $G_{\alpha i}$ -GDP, by controlling dynamically the cycling of $G_{\alpha i}$ from a GDP to a GTP form. However, an important factor in this scenario would be the identity of a Frizzled receptor on the lateral side of developing HCs. Fz3 and Fz6 have been shown to be expressed on the medial pole of HCs but do not have a classical PDZ-BM, and remain asymmetrically localized

in *Daple* mutants (as is *Vangl2*), whereas to date, no Frizzleds are known to be expressed on the lateral sides (Ezan and Montcouquiol 2013). Messenger RNAs (mRNAs) for several Frizzleds, including *Fz1*, 2, 4, and 9, have been reported to be present in the cochlea but localization of protein has not been examined because of a lack of appropriate antibodies. Further, the phenotypes of *Dvl* (or *Fz*) mutants differ from *Daple* mutants, both in terms of hair bundle orientation and cochlear extension, suggesting more complicated interactions between the two proteins that could include roles for different *Dvls* (possibly mediated by *Daple*) in both tissue PCP and cell-autonomous polarization.

An additional consideration is the physical localization of the different components. *Gpsm2*, $G_{\alpha 3}$, aPKC, and PAR6 are all located in the apical HC membrane and at the level of TJs, whereas the core PCP protein *Vangl2* is located more basally in the adherens junction region (Ezan et al. 2013). *Daple* appears to be somewhat overlapping but mostly below the TJ (based on ZO-1 labeling), whereas *Dvl2* is below *Daple*, and none of them are at the apical membrane (Siletti et al. 2017; Sipe et al. 2013). Although the detection of all of these proteins is challenging, and an absence of staining does not prove an absence of expression, it will be important to determine the degree of physical overlap in developing HCs.

ORIENTED CELL DIVISION AND HAIR CELL PCP: A CONSERVED MOLECULAR TOOLBOX

The Par family of cell polarity proteins (Par3/Par6/aPKC) plays prominent roles in regulating the formation and maintenance of apicobasal (A/B) polarity in epithelial cells (Tepass et al. 2001; Hirose et al. 2002; Rolls et al. 2003; Hutterer et al. 2004). In a typical polarized epithelial mammalian cell, the aPKC–Par3–Par6 complex has an asymmetric apical distribution, with Par3 distinctly localized to TJs, whereas aPKC/Par6 distinctly localized to TJs and aPKC/Par6 are localized more apically (above the TJ on the apical surface) (Hirose et al. 2002; Harris and Peifer 2005; Martin-Belmonte et al. 2007; Bryant and

Mostov 2008; Morais-de-Sá et al. 2010). However, the Par proteins were originally identified in *Caenorhabditis elegans* as regulators of asymmetric cell division (Kemphues et al. 1988; Kemphues 2000). Par3, aPKC, and Par6 transiently accumulate asymmetrically during the oriented division of cells in at least three major systems (and many more): the one-cell embryo of *C. elegans*, the neuroblasts (NBs) of the *Drosophila* central nervous system, and the sensory organ precursors (SOPs) of the peripheral nervous system. Pins and its binding partner, $G_{\alpha i}$, do not appear to be required for A/B polarity but interact with the Par complex during cell division to provide important asymmetric cues for the attachment and the control of the position of the mitotic spindle during mitosis.

During *Drosophila* NB division, Bazooka (Baz, Par-3 in other species), Par6, and aPKC act as an apical cue inherited from the epithelium to establish asymmetry by recruiting Insc via binding to Par3, which in turn recruits Pins to the apical side of the NB (Fig. 4D). Binding of Insc to Pins triggers a conformational switch enabling the relocation of $G_{\alpha i-GDP}$ into the complex (Schober et al. 1999; Wodarz et al. 1999; Schaefer et al. 2000, 2001; Yu et al. 2000). The tetratricopeptide (TPR) domains of Pins can then associate with the microtubule-binding protein mushroom body defect (Mud in invertebrates)/NUMA in vertebrates). The binding of Insc to Pins appears to be exclusive to its interaction with Mud/NUMA, resulting in a shift from a Par/Insc/Pins/ $G_{\alpha 3}$ recruiting complex to a Pins/ $G_{\alpha i}$ /NUMA pulling complex (Culurgioni et al. 2011). The Pins/ $G_{\alpha 3-GDP}$ /NUMA complex is required to capture astral microtubules, orient the mitotic spindle, and exert pulling forces, via binding to the microtubule motor dynein/dynactin, which provides a sliding anchorage for depolymerizing microtubules, whose shrinkage pulls the connected spindle pole toward the cortex (Schober et al. 1999; Wodarz et al. 1999; Kaltschmidt et al. 2000; Schaefer et al. 2000; Yu et al. 2000). The pulling complex is also comprised of Lis-1 and p21-activated kinase (PAK) (Siller and Doe 2008; Bompard et al. 2013), which appears to be conserved in HCs as recently reviewed by Lu and Sipe (2016).



An alternative and partially redundant Insc/Par-independent pathway was also described in the *Drosophila* NB. In this case, an asymmetrical Pins/Dlg/Khc73 complex made of the plus-end-directed microtubule motor protein kinesin heavy chain 73 (Khc-73 or GAKIN in mammals), the membrane-associated guanylate kinase (MAGUK) protein, and the tumor-suppressor protein Discs large (Dlg) forms (Fig. 4C) (Siegrist and Doe 2005). Dlg directly binds Pins after the phosphorylation of Pins by the mitotic kinase Aurora-A (Johnston et al. 2009), and then binds to and activates Khc73. The Dlg/Khc73 complex contributes to a microtubule-induced establishment of Pins polarity, while polarized Pins/Dlg/Khc73 serve as a capture site for dynamic astral microtubules. Interestingly, imaging of the cochlea of a transgenic mouse line that constitutively expresses a Venus fluorescent protein fused to the amino terminus of Dlg3 showed a GFP-tagged abneural crescent, similar to Ins/Gpsm2-LGN/ G_{ci} , suggesting that a similar mechanism is also at play during kinocilium migration and bundle maturation (Gegg et al. 2014). However, mutation of another member of the Dlg family, Dlg-1, resulted in very mild (if any) hair bundle defects in the cochlear epithelium, suggesting functional redundancy (Rivera et al. 2013). The absence of reliable antibodies for Dlg-1 prevents confirmation of this hypothesis at the moment.

Regarding Par3, discrepancies in its localization have been reported (Ezan et al. 2013; Tarchini et al. 2013). Abneural location together with Insc would suggest a NB-like mechanism with a Par3/Ins/Pins/ G_{ci} complex acting on microtubules. However, in NBs, Par3 (together with Par6/aPKC) colocalize with Pins/ G_{ci} , something that was not observed in HCs by either group. Instead, in HCs, Gpsm2/ G_{ci} are localized opposite to aPKC/Par6 in a pattern that is similar to that which occurs in SOPs (Fig. 4E). In this context, G_{ci} can localize directly to the plasma membrane through myristylation (Cho and Kehrl 2008; Gönczy 2008; Thyagarajan et al. 2011). Also, *Drosophila* SOP cells are specified in a polarized epithelium, but while they divide asymmetrically they ignore the A/B polarity from the epithelium and instead orient along a

PCP axis (Lu et al. 1999; Bellaïche et al. 2001). The SOP planar mitotic spindle orientation then depends on and responds to core PCP protein asymmetry such as Frizzled (Fz)/Dishevelled (Dsh/Dvl) colocalizing with Baz-Par3/aPKC/Par6, whereas Strabismus/van Gogh (Stbm/Vang) and Prickle (Pk) colocalize with Pins/ G_{ci} on the opposite side (Goodrich and Strutt 2011; Morin and Bellaïche 2011). This is similar to the OC, with an already established A/B polarity and the presence of early core PCP cues (Montcouquiol et al. 2003; Ezan and Montcouquiol 2013). More recently, F. Schweisguth's group showed that core PCP signaling is the initial symmetry-breaking signal for the planar polarization of Par proteins in SOPs, suggesting that the epithelium becomes planar polarized at early stages, after which SOP fate is determined allowing Baz, Par6, and aPKC to read the PCP information from the tissue and to asymmetrically localize (Besson et al. 2015). A similar model has been proposed in the cochlear epithelium where in the absence of PCP signaling the kinocilium can migrate to the abneural side of the HC (cell-autonomous PCP), but the coordination of the orientation of the HC within the tissue (non-cell-autonomous or tissue PCP) is disrupted because the Par complex is missing global core PCP cues (for review, see Ezan and Montcouquiol 2013).

Altogether, these observations show that the HC has remarkably adapted the molecular machinery used during orientated cell division of invertebrates to mediate a different developmental event, the polarization of a structure, the stereociliary bundle, in a postmitotic cell.

PCP, CILIA, AND TRAFFICKING

Defects in kinocilia migration and bundle formations have also been observed in the inner ear epithelia of targeted mouse mutants for three intraflagellar transport (IFT) genes (Jones et al. 2008; Sipe and Lu 2011; May-Simera et al. 2015). Flattened or misshapen/circular bundles that can be separated from the kinocilia are also observed in mouse mutants for genes implicated in human ciliopathies, which include BBS, MKS, and Alms (May-Simera 2016). In addition, mu-

tations in *Mkks* or *Bbs8* affect the apical distribution of $G_{\alpha i}$ (Ezan et al. 2013; May-Simera et al. 2015). This suggests a link between the basal body, or the machinery around it, and the targeting of $G_{\alpha i}$ at the apical membrane. Consistent with this, robust protein trafficking is observed at the base of cilia in all cells, including HCs, as illustrated by the accumulation of Rab11 in the basal body area of the kinocilium (Kirjavainen et al. 2015). The importance of trafficking machinery (regardless of its relation to the kinocilium) for PCP was recently emphasized by the work of Jagger and colleagues, using a *Sorcs2* (*Sortilin-related VPS10 domain-containing receptor 2*) mutant (Forge et al. 2017). *Sorcs2* belongs to a family of proteins involved in protein trafficking and intracellular/intercellular signaling with a primary role in the sorting of cargos from endosomes to the *trans*-Golgi network or to the cell surface (Reitz 2015). The phenotypes observed in *Sorcs2* mutants are similar to, but more severe than those of *Gpsm2* or $G_{\alpha i}$ mutants, including misplacement of the kinocilium or abnormalities in bundle morphology. Critically, disruption of *Sorcs2* expression leads to an almost complete absence of *Gpsm2*-LGN and $G_{\alpha i3}$ in the apical domain of the HC, while aPKC is no longer distributed asymmetrically. The punctate and vesicular immunolocalization of the protein is consistent with a role of *Sorcs2* in the directed sorting of *Gpsm2*-LGN/ $G_{\alpha i}$ to the apical domain of the HC. Altogether, these results show that the establishment of PCP within the inner ear epithelia requires the coordination and the integration of complex molecular mechanisms including A/B polarity, trafficking, and cilium-related machinery.

GPSM2-LGN/ $G_{\alpha i3}$ SIGNALING: ADDITIONAL ROLES BEYOND MICROTUBULE REGULATION

In humans, mutations in *GPSM2* (OMIM# 604213) result in a rare neurological disorder called Chudley-McCullough syndrome (CMCS), which is associated with congenital-to-prelingual deafness and developmental brain anomalies (Doherty et al. 2012). Similarly, mice with a targeted mutation in *Gpsm2* are profoundly deaf

at P17 (Bhonker et al. 2016). Two recent studies showed that the observed auditory deficits and the neurological phenotypes in both patients and mouse mutants are probably not a result of the classical, tubulin-dependent role of the *Gpsm2*/ $G_{\alpha i3}$ complex (Tarchini et al. 2016; Mauriac et al. 2017).

By generating different conditional mutants for both *Gpsm2* and $G_{\alpha i3}$, these studies showed that the elongation of the actin-rich stereocilia in the cochlear and the vestibular system, in particular those of the IHCs, is inhibited in the absence of *Gpsm2*, $G_{\alpha i3}$, or, more surprisingly, when all three $G_{\alpha i}$ s were blocked through expression of pertussis toxin PTX in HCs. As a result, mechanotransduction is defective in these HCs leading to a disruption in hearing and balance. Remarkably, this result was confirmed in vitro using pertussis toxin treatments to block $G_{\alpha i}$ function (Mauriac et al. 2017). The studies further showed that both *Gpsm2* and $G_{\alpha i3}$ proteins accumulate at the tip of the longest stereocilia, before initial elongation (Fig. 4C). This “tip domain” is a small region at the extremity of the stereocilium where elongation occurs by the addition of actin monomers at the barbed ends (plus ends) of the filaments (Tilney et al. 1981). Mauriac et al. identified the adaptor Whirlin (a member of the Usher protein network) as a new *Gpsm2* interactor that binds to the amino-terminal region of the protein, and establishes a complex that also includes unconventional Myosin15 and Eps8 (Mauriac et al. 2017). This complex is required for the transport of *Gpsm2* and $G_{\alpha i3}$ to the tip of the stereocilia but does not play a role in the earlier localization of *Gpsm2*/ $G_{\alpha i3}$ to the fonticulus (Mauriac et al. 2017). Importantly, *Insc* does not appear to participate in this mechanism (Tarchini et al. 2016). These results show that the targeting of *Gpsm2*/ $G_{\alpha i3}$ proteins depends on different interactions and protein complexes for fonticular versus stereociliary localization.

Overall, these results show a surprising new role for *Gpsm2*/ $G_{\alpha i}$ in the regulation of actin polymerization. Mauriac et al. (2017) also suggest that a similar molecular mechanism for controlling of actin dynamics via *Gpsm2*/ $G_{\alpha i}$ exists in neurons, modulating the actin retrograde actin flow and neuronal outgrowth, sug-



gesting a much broader role for these proteins in cytoskeletal remodeling.

SUMMARY

The abilities of individual cells or tissues to generate asymmetry, either within a cell or across a group of cells, play crucial roles across most developmental processes. Several striking examples of these asymmetries occur during the development of the inner ear. In this article, we have highlighted two of these processes, the active outgrowth and concomitant patterning of the cochlear duct and the formation and orientation of the asymmetric stereociliary bundle of the HCs. Cochlear outgrowth occurs through a complex process of cellular intercalation, convergence, and growth. Although the molecular mechanisms that mediate these processes are still poorly understood, the core PCP pathway plays an instructive role in this process, while many of the mechanical changes in cell position or shape are dependent on Myosin II. The core PCP pathway is also known to play a role in the uniform orientation of stereociliary bundles across the entirety of different HC epithelia, including the cochlea, but, in contrast with its role in *Drosophila*, does not play a role in the specification of asymmetry within an individual cell. Instead, recent work from two laboratories has shown that a conserved signaling pathway originally identified through its role in the orientation of mitotic spindles regulates both the asymmetric localization of the developing kinocilia, the shape of the hair bundle, and, more surprisingly, the subsequent elongation of stereocilia. These results provide new insights regarding the factors that regulate asymmetry, polarity, growth, and patterning during cochlear development while also elucidating the etiologies of genetically based inner ear disorders, including Chudley–McCullough syndrome and Mondini dysplasia.

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