Non-cell-autonomous planar cell polarity propagation in the auditory sensory epithelium of vertebrates

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ABSTRACT

Sensory epithelia of the inner ear require a coordinated alignment of hair cell stereociliary bundles as an essential element of mechanoreceptive function. Hair cell bundle alignment is mediated by core planar cell polarity (PCP) proteins, such as Vangl2, that localize asymmetrically to the circumference of the cell near its apical surface. During early phases of cell orientation in the chicken basilar papilla (BP), Vangl2 is present at supporting cell junctions that lie orthogonal to the polarity axis. Several days later, there is a striking shift in the Vangl2 pattern associated with hair cells that reorient towards the distal (apical) end of the organ. How the localization of PCP proteins transmits planar polarity information across the developing sensory epithelium remains unclear. To address this question, the normal asymmetric localization of Vangl2 was disrupted by overexpressing Vangl2 in clusters of cells. The BP was infected with replication-competent retrovirus encoding Vangl2 prior to hair cell differentiation. Virus-infected cells showed normal development of individual stereociliary bundles, indicating that asymmetry was established at the cellular level. Yet, bundles were misoriented in ears infected with Vangl2 virus but not Wnt5a virus. Notably, Vangl2 misexpression did not randomize bundle orientations but rather generated larger variations around a normal mean angle. Cell clusters with excess Vangl2 could induce non-autonomous polarity disruptions in wild-type neighboring cells. Furthermore, there appears to be a directional bias in the propagation of bundle misorientation that is towards the abneural edge of the epithelium. Finally, regional bundle reorientation was inhibited by Vangl2 overexpression. In conclusion, ectopic Vangl2 protein causes inaccurate local propagation of polarity information, and Vangl2 acts in a non-cell-autonomous fashion in the sensory system of vertebrates.

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Introduction

Planar cell polarity (PCP) refers to a systematic, yet asymmetric, orientation of cells or subcellular structures across the plane of an epithelial sheet. Prominent examples of PCP are wing bristles and ommatidia of the fruit fly, Drosophila, and the stereociliary bundles on the apical surfaces of hair cells in the vertebrate inner ear. Studies of fruit fly and mouse mutants have revealed a shared set of core PCP components that are required during ontogeny to establish asymmetry (reviewed by Wu and Mlodzik, 2009). For example, Vangl2 (also called cLtap) is the vertebrate ortholog of Drosophila's core PCP gene Stbm/Vang. A spontaneous mutation in Vangl2 (Loop-tail, Lp mice) leads to severe hair cell orientation defects in mouse inner ears (Montcouquiol et al., 2003). Defective hair cell orientation is also caused by mutations in PCP genes coding for Celsr1, Fz3/6, and Dvl1/2 (reviewed by Goodrich, 2008).

Morphologically, stereocilia initiate as microvillar protrusions that surround a centrally located non-motile kinocilium on the apical surface of an immature hair cell (Fig. 1A). The kinocilium then moves to the periphery, accompanied by differential elongation of the microvilli into stereocilia. The stereocilia elongate into rows of increasing height, with the tallest row located closest to the kinocilium. In the auditory epithelia, the kinocilia initially all face laterally (mammals) or abneurally (birds) to achieve a rough global alignment (Fig. 1B). A second shift in polarity, which is called “reorientation” (Cohen and Cotanche, 1992) (Fig. 1C), occurs in the basilar papilla (BP) of many birds, but not in the mammalian cochlea. Reorientation is most prominent in the distal two-thirds of the organ, where centrally-located hair bundles turn up to 90° towards the apex (reviewed by Gleich and Manley, 2000). The functional correlation of this systematic reorientation, which is apparently unique among tetrapods, is unknown. Finally, the orientation of each bundle is more precisely adjusted, in a phase we call “refinement”, resulting in lower variation within the population (Fig. 1D).

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Before morphological asymmetries are evident, core PCP proteins such as Vangl2 and Pk2 localize asymmetrically at the sub-apical membrane of one cell side in the mouse (Deans et al., 2007; Qian et al., 2007). The chicken homolog of celsr-1, c-fmi-1, shows a polarized expression on both sides of hair cells (Davies et al., 2005). How PCP protein localization mechanistically translates into hair cell orientation is unclear. Independently, there is evidence that ciliary proteins (ff88) are necessary to direct the kinocilium (Jones et al., 2008). Furthermore, ciliary genes involved in the Bardet–Biedl syndrome, such as Bs1 and Mksks, genetically interact with Vangl2 (Ross et al., 2005).

Insight into the cellular autonomy and propagation of PCP information across an epithelium has relied on genetic mosaic approaches conducted largely in Drosophila (reviewed by Lawrence et al., 2007) but also recently in Xenopus larvae (Mitchell et al., 2010). We devised an analogous approach using virus-mediated gene misexpression to create clusters of Vangl2 overexpressing cells embedded among wild-type cells in the chicken inner ear. This method revealed that Vangl2 misexpressing cells influence the polarity of their wild-type neighbors, thus demonstrating that polarity is propagated from cell to cell in a vertebrate sensory organ. Notably, the reorientation phase of bundle polarity was unable to proceed normally when Vangl2 protein was overexpressed.

Materials and methods

Retrovirus preparation and injection

The replication-competent avian retroviral vector, RCASBP(A)/Ltap, contains an insert (1600 bp) encoding chicken Ltap full-length open reading frame (Kibar et al., 2001) and is referred to below as RCAS/Ltap. RCASBP(A) parent virus with no insert served as a control (called RCAS). Plasmid encoding RCASBP(A)/Wnt5a virus (called RCAS/Wnt5a) was published previously (Hartmann and Tabin, 2000). Pathogen-free fertilized chicken eggs were received from ATCC, CRL-12203) with pRCAS vectors was followed by collection of supernatants from virus-infected DF-1 cells as follows. DF-1 cells were infected with 10 μl of concentrated RCAS or RCAS/Wnt5a in growth medium of 10% fetal calf serum (Atlanta Biologicals), 2% chicken serum (Sigma) in DMEM (Sigma). After 7 days of passaging, the cells were allowed to approach confluence and growth medium was reduced to half volume 24 h before collection of the supernatant (i.e., the conditioned medium). A total of 0.5 ml of conditioned medium was added to each well.

For analysis of axon outgrowth, gels were fixed with 4% paraformaldehyde in PBS, washed and immunostained with mouse anti-β-tubulin (IgG2b, 1:500, Sigma) followed by AlexaFluor 488 goat-anti-mouse (Molecular Probes). Confocal images were sampled every 10 μm and then collapsed as 2D projections that were imported into NIH ImageJ 1.4 (open source software, Rasband WS, US NIH, Bethesda, MD: http://rsb.info.nih.gov/ij/). Pixels occupied by axons emanating from the floor plate into the gel were thresholded and quantified per unit of longitudinal explant length (μm).

Tissue preparation and immunohistochemistry

In addition to virus-treated embryos, untreated embryos were harvested at E10 to E18 as controls and for a developmental time course of Vangl2 expression. The embryonic cochlear duct was dissected from surrounding temporal bone and the BP was separated from the auditory nerve, the abneural limbus and the tegmentum vasculorum. The exposed epithelium was stored in PBS (with 0.05% sodium azide as preservative) until whole mount staining. In addition,
a subset of intact cochlear ducts was embedded and sectioned at 15 μm in preparation for immunostaining.

Filamentous actin (F-actin) of hair cell bundles was visualized with AlexaFluor568 phallolidin conjugate (1:1000, Invitrogen). A mouse monoclonal antibody against acetylated α-tubulin (IgG2b, 1:500, Sigma) aided the discrimination of hair cell bundle sides either by labeling the kinocilium if present and/or by demarcating the side of the tallest row of stereocilia because tubulin protein (α- and β-tubulin) is expressed asymmetrically on that side (see Results). Virus infection was monitored using AMV-3C2 hybridoma supernatant (IgG1, 1:20) prepared from cells purchased from Developmental Studies Hybridoma Bank (DSHB, University of Iowa, USA), The monoclonal 3C2 antibody is directed against an epitope of the viral gag-encoded matrix protein, MA, also known as p19 (Potts et al., 1987). Vangl2 protein detection utilized a rabbit polyclonal anti-Vangl2 (1:300) (Montcouquiol et al., 2006; Warchol and Montcouquiol, 2010; Zhang and Levin, 2009).

Appropriate goat-anti-mouse or goat-anti-rabbit AlexaFluor-conjugated (AlexaFluor488, AlexaFluor647) secondary antibodies were applied in addition to AlexaFluor568 phallolidin. An independent set of specimens was used to confirm virus infection efficiency by staining E10–E11 tissue sections with 3C2 antibody and biotinylated goat-anti-mouse secondary antibody (IgG H+L, 1:250, Vector Laboratories) with extravidin–biotin–horseradish peroxidase complex (1:200, Vector Laboratories ABC Standard Kit). Peroxidase activity was revealed using diaminobenzidine histochemistry. Sections were dehydrated and coverslipped.

Blocking and antibody incubations were carried out at 4°C in either 10% heat-inactivated calf or goat serum with 0.05% TritonX100 (0.5% TritonX100 for whole mounts), 0.05% sodium azide in PBS overnight. The first antibody reactions took place overnight or up to 3 days (in the case of anti-Vangl2), and extensive washes with PBS were followed by secondary antibody reactions along with phallolidin conjugate for 3 h. BPs were mounted using spacers (to prevent crushing) in Vectashield soft-set mounting media (Vector Laboratories). Immunofluorescent sections were cover-slipped with Vectashield hard-set mounting media (Vector Laboratories) and imaged with a Spot Flex Color CCD camera (Diagnostics Instrument, Inc., Sterling Heights, MI) attached to a Nikon E800 microscope.

Confocal microscopy and image analysis of BP whole mount preparations

Confocal fluorescence microscopy (BioRad MRC1200) was used to acquire images for quantitative measurements of hair cell bundle orientation. First, an overview image montage of the entire auditory epithelium (BP) was compiled for each analyzed BP by image stitching of 5–8 low magnification confocal image stacks taken along the length of the BP (Pages 4.0, Apple Inc.). Measurements of organ length and width were gathered with image analysis tools in NIH ImageJ 1.4. Second, confocal image stacks of analysis areas were taken at high magnification. Image stacks with 20–50 confocal slices at z-axis intervals of 0.5–2.5 μm were collapsed to obtain a z-projection. Analysis area images were then mapped back to the overview by superimposing matching cells on the montage (Fig. 2A–C). This allowed us to extrapolate the coordinates of each analysis area as percentage from the neural BP edge (width coordinate) and percentage from the base (length coordinate). These coordinates were used to target an approximately matching pair of analysis areas in both the control (left) and the corresponding experimental (right) BP of an embryo. At each analysis position, a tangent line to the neural BP edge defined the base line for bundle orientation angle measurements (Fig. 2A, B, D). Data for left and right ears were normalized. Measurements were carried out with NIH ImageJ 1.4 and organized with Numbers 2.0 (Apple Inc.). For further statistical analysis, StatPlus 5.7 (AnalystSoft Inc.) was utilized and graphs were produced with DataGraph 2.1 (VisualDataTools, Inc.).

Results

Orientation of hair cell bundles in the developing BP

During ontogeny, planar polarity is observed shortly after the initiation of stereocilia bundle formation in the BP (Cohen and Cotanche, 1992). In whole mounts, phalloidin-labeling reveals the bundles, while the asymmetric expression of tubulin adjacent to the tallest row of stereocilia (Fig. 2E) defines the front face of the bundle. Bundles oriented parallel to the organ edge and facing abneural are designated an orientation angle of 180° (Fig. 2D), following an established convention (Cotanche and Corwin, 1991). Bundles rotated towards the apex versus the base are assigned angles of greater than and less than 180°, respectively (Fig. 2D).

By E12–14, hair cells in most areas of the BP are roughly aligned and initially face abneurally (Supplemental Fig. S1A,B). Reorientation of hair cells takes place progressively and occurs mainly in central areas of the distal two-thirds of the BP. Refinement leads to hair cell orientation angles that vary systematically with position (from base to apex and over the organ width). In the mid-proximal BP (20–25% length distance from the base), the average angle confines to 182° ± 10° (n = 3 ears; 224 hair cells) at the age of E14–15 (Supplemental Fig. S1C). A ± 10° standard deviation is consistent with published data for the mid-proximal region (Cotanche and Corwin, 1991).

Vangl2 protein expression in the developing auditory epithelium

In the immature BP, each hair cell is surrounded by a rosette of supporting cells. At E12, supporting cells have a broad apical surface (Fig. 3A, A') that subsequently narrows, allowing the growing hair cells to get closer together (Fig. 3B, E14). At young stages, it is relatively easy to distinguish between cell membranes of hair cells versus supporting cells and to visualize contacts between adjacent supporting cells. We examined Vangl2 immunoreactivity on whole mount BP preparations following an abbreviated tissue fixation protocol (time of exposure to fixative was limited to 20–25 min). The developmental time course reveals that supporting cells express Vangl2 at some but not all of the junctions with adjacent supporting cells. Vangl2 expression is strongly biased to the junctions that lie orthogonal to the lateral sides of the hair cells (i.e., the sides flanking the developing stereocilial bundle) at E12–14 (Fig. 3A, A', and B, B'). Columns of supporting cells thus resemble a series of ladders spanning the epithelial sheet, with the rungs expressing Vangl2.

In z-projection confocal image stacks, there are obvious crescent-shaped expression foci of Vangl2 associated with hair cells at later developmental stages, for example, at E17 (Fig. 3C), when the enlarged apical surfaces of the hair cells largely supersede those of supporting cells. However, it is difficult to ascertain whether these crescents are expressed within the hair cells proper or rather at the subapical membrane of the adjacent supporting cell processes, which angle strongly from abneural towards neural as they project upward to reach the apical surface. In either case, the crescent is present asymmetrically on the neural side of hair cells (opposite to the kinocilium) in proximal BP sample areas (Fig. 3C). Sample areas located in the central region of the epithelium, in particular in the distal BP, also display Vangl2 crescents but these have shifted by 90° compared to more basal sample areas (Fig. 3D).

Thus, a shifted Vangl2 protein localization is observed in those regions of the BP where hair cells have reoriented towards the distal tip of the organ.
Virus infection induces Vangl2 overexpression in cell clusters

Retroviral vectors encoding Vangl2 (RCAS/cLtap) were injected into the right otocyst on E3 at ~10^5 infectious units per otocyst. Mitotically-active otic progenitor cells will be infected at random, integrate the viral genome, and transmit it to all of the progeny of one of their two daughter cells. Since the virus is replication-competent, it can also spread to unrelated cells that are actively dividing. The majority of hair cells and supporting cells withdraw from the cell cycle between E5 and E7 in the middle regions of the BP and the entire organ is largely quiescent by E8.5 (Katayama and Corwin, 1989). Because virus-mediated protein expression is detectable within 10 h and is robust by 24 h after virus injection (Homburger and Fekete, 1996), we expect that many infected sensory cells will begin to express the Vangl2 transgene while they are still mitotic. Infected cells will continue to express the transgene after they differentiate. Thus, Vangl2 misexpression begins prior to, and continues throughout, all three PCP phases: hair bundle orientation, reorientation and refinement.

Infection with RCAS/cLtap results in defects in hair cell bundle orientation that are obvious when comparing surface preparations of the injected ears to the contralateral controls (Fig. 4C, D) or to ears infected with RCAS/Wnt5a (Supplemental Fig. S3A–C). Bioactivity of the RCAS/Wnt5a virus stock was confirmed by its ability to enhance axon outgrowth in cultured chicken spinal cords (Supplemental Fig. S3D–F). Immunolabeling with anti-Vangl2 following overnight fixation reveals ectopic, but not endogenous, expression of Vangl2 protein, possibly due to epitope masking (compare Fig. 4E, F and G, H). Exogenous Vangl2 expression is readily detectable in RCAS/cLtap-infected specimens because the raised Vangl2 protein in infected cells extends throughout the cytoplasm (Fig. 4F). A subset of samples was subjected to triple-labeling with anti-Vangl2, 3C2 and phalloidin. This method reveals individual hair cells with a defective bundle alignment, expression of viral gag-epitope and apparent overexpression of the Vangl2 protein in experimental ears but not controls (Fig. 4G, H). Individual hair cells, as well as supporting cells, in RCAS/cLtap papillae were immunopositive for cytoplasmic Vangl2; these cells were situated among sensory epithelium cells that do not overexpress the PCP protein, creating a mosaic expression (Fig. 4I).

Overexpression of Vangl2 leads to disrupted hair cell orientations

The effect of RCAS/cLtap virus on hair cell orientation was evaluated based on comparisons between the un.injected left (control) ear and the RCAS/cLtap injected right (experimental) ear.
of the same embryo. Hair cell bundle assembly and morphology, as well as the mosaic of hair cells and supporting cells, are mostly unaffected in RCAS/cLtap BPs (Fig. 4H). However, Vangl2 over-expression clearly disrupts hair bundle orientation (Fig. 4B, D, H). Quantification of hair bundle orientations was performed at three random locations per BP in the proximal two-thirds of the epithelium and compared with three approximately matching locations per control BP. Ten embryos yielded a total sample size of 60 analysis areas (Supplement Table S1). On average, sample areas are located at 35% length distance from the BP base (proximal) and 60% from the neural edge. The average (mean of means) hair cell orientation angle of controls is 195º with a standard deviation of ±12º ($n=3316$ cells) for the 30 sample areas. RCAS/cLtap-infected ears display an average hair cell orientation angle that is similar to controls at 189º, but with much higher variation: their standard deviation is ±27º ($n=3025$ cells).

For the purpose of statistical comparisons, independent sampling is obtained by evaluating only one of the three matched pairs of analysis areas per embryo. These 20 samples are indicated by asterisks in Supplement Table S1 and are compared in Fig. 5A. It is obvious from the individual histograms that there is a larger variance of polarity angles in RCAS/cLtap papillae versus controls. Neither the mean nor the median hair cell orientation angles are significantly different between control and experimental data sets (Supplemental Fig. S4).

The population distributions of all cells from these 20 sample areas are plotted in Fig. 5B. Tests of normality for these populations showed
that control orientations follow a normal distribution according to Lilliefors (no evidence against normality) and Shapiro–Wilk tests (null hypothesis rejected at 5% level, \( p = 0.0706, n = 986 \) cells) (pink histogram Fig. 5B). Experimental orientations do not assume a perfect normal distribution (blue histogram Fig. 5B). While the Lilliefors test indicates no evidence against normality, the Shapiro–Wilk test rejects normality, \( p = 0.0027, n = 922 \) cells).

Statistical analysis indicates that control and RCAS/cLtap hair cell orientations differ significantly in their variance. For control hair cells, the interquartile range (IQR) is 17 whereas the experimental IQR equals 47 (Fig. 5C). Furthermore, significance in variance between control and experimental orientation angles is confirmed by descriptive two-sample \( F \)-Test for variance where the probability corresponding to Fisher criterion, is zero with \( F \)-critical value 0.1439. Non-parametric comparisons indicate a difference with a high level of significance (Kolmogorov–Smirnov test \( p = 0 \); Mann–Whitney \( U \) test \( p = 0.0264 \); and Wald–Wolfowitz Runs test \( p = 0 \)).

Hair cell bundles are not randomly oriented in response to Vangl2 overexpression

In RCAS/cLtap papillae, a rudimental epithelial polarity persists, as evidenced by a strong bias in bundle orientation despite a significant increase in variance from the average. That is, the orientation patterns of experimental epithelia are not randomized. Comparison of the experimental data set \( n = 922 \) cells against a uniform distribution utilizing non-parametric Mann–Whitney \( U \) test \( p = 0.0001 \) and Wald–Wolfowitz Runs Test \( p = 0.0004 \) indicates a highly significant difference between RCAS/cLtap orientations versus random orientations.

**Fig. 4.** Virus infection and ectopic Vangl2 protein expression in control and RCAS/cLtap basilar papillae (BPs). A–H: Matching pairs of the contralateral (control, left) and injected ipsilateral (experimental, right) BP of the same embryos. A–I: F-Actin (red) labels hair cell bundles and cell borders. Image pairs of surface view whole mount BP preparations are oriented with abneural to the top and the apex towards the right (RCAS/cLtap panels are mirror-image reversed for comparison to control). A, B: RCAS virus gag epitope labeling with 3C2 antibody in confocal \( z \)-projections of sample areas of whole mount BPs (E16). (Sample location of control: 42% length (L) distance from BP base, 62% width (W) distance from the neural edge; sample location of RCAS/cLtap: 36% L, 70% W). 3C2 reactivity (blue). C, D: Hair cell orientation patterns of sample areas of whole mount BPs (E17). The control (C) was sampled from BP location 25% L, 47% W and the RCAS/cLtap injected BP (D) was sampled from 26% L, 53% W. E, F: Detection of excess Vangl2 protein (green) in BP cross sections of 15 \( \mu \)m thickness, (E18). G, H: Triple labeling of F-actin (red), excess Vangl2 protein (green) and gag viral epitope (3C2, blue) in whole mount preparations, (E19–20). I: Patches of excessive Vangl2 expression (green) in an overview confocal image montage of an experimental BP whole mount (mid-proximal region), (E18). E, embryonic day. Scale bar = 20\( \mu \)m.

**Fig. 5.** Comparisons of control (pink) versus RCAS/cLtap (blue) hair cell orientations. Data represent analyses of one sample area per BP (see asterisks in Supplemental Table S1). A: Histograms of bundle orientation angle frequencies from matched pairs of sample areas of 20 individual basilar papillae (BPs). B: Scatter graph and histogram of bundle orientation angles of 10 control and 10 RCAS/cLtap BPs. On the scatter graph, the Y-axis refers to an arbitrary number assigned to each cell. C: Whisker box plots of control (contr, \( n = 986 \) cells) and experimental (exp, \( n = 922 \) cells) orientation angles from one location per BP.
Importantly, cells with a full reversal of bundle orientation (or a value of -0º/360º) were not observed in any RCAS/cLtap papilla, as might be expected if either no polarity information remains for the hair cell to read out or wild-type cells were reversed due to confrontation with an infected neighbor displaying ectopic Vangl2 on the “wrong” side. Among all analyzed samples, we found a maximal range of orientation angles in controls between the extremes of 140º and 281º (n = 3316). The most extreme angles measured from experimental BPs were 63º and 330º (n = 3025). Thus, cells never populated a segment of about 90º in orientation angles that represents the opposite quadrant to the normal mean angle.

Vangl2 misexpression induces non-cell-autonomous polarity defects

Immunoreactivity of endogenous membrane-associated anti-Vangl2 decreased as the duration of tissue fixation was lengthened; thus we used overnight fixation to obtain selective labeling of Vangl2 overexpression throughout the cytoplasm (Fig. 6A, B). This is utilized to address how hair cell orientations are affected both within and beyond infected patches. Comparison of control versus experimental ears shows that PCP is indeed disrupted in sensory epithelia that have Vangl2 overexpression (Fig. 6C, D). In a control ear, only 4–5% of the hair cells deviate from the average alignment of their neighbors (Fig. 6C, bundles shown as red bars), as determined by an orientation angle beyond 2 standard deviations (± 2σ) from the mean of a normal distribution. The comparable area of the contralateral RCAS/cLtap papilla shows many more cells with bundles angled greater than ± 2σ from the mean of the control (Fig. 6D, red bars). In the infected ear, the misaligned bundles are scattered among bundles that are within the normal range (Fig. 6D, blue), which is operationally defined as less than ± 2σ from the mean of the control ear. We note that all cells within an overexpressing cell cluster (green) are not misaligned, nor can we detect exogenous Vangl2 protein in all misaligned cells (Fig. 6B, D).

An advantage of virus-mediated gene transfer is that only about half of the sensory cells are infected following E3 injections at the titers used here. Because the virus only integrates into mitotically active cells (Roe et al., 1993), it does not spread to all sensory cells before they exit the cell cycle between E5 and E7 in the middle regions of the BP (Katayama and Corwin, 1989). Non-cell-autonomous changes in bundle polarity can thus be detected by asking whether infected cells influence the alignment of their wild-type neighbors. Furthermore, we can determine whether there is a directional bias, i.e., whether the location of affected cells relative to the misexpression cell cluster is of relevance (schema Fig. 7A).

Indeed, aggregate data from two sample areas of different embryos reveal a highly significant correlation between Vangl2 overexpression and orientation defects (greater than ± 2σ from the mean angle of controls). This correlation is confirmed by nonparametric 2×2 table analysis. In detail, of all cells within a misexpression patch, the majority (40 vs. 14 cells) has a disturbed orientation and an equal number of cells adjacent to a Vangl2-patch display a misaligned or a normal orientation (97 vs. 98 cells) (Fisher’s Exact Test, p = 0.001, n = 249 cells) (Fig. 7B). Among all disturbed cells, the vast majority is...
patch-related, defined as being situated either within or adjacent to a Vangl2-patch (137 vs. 33 cells). In contrast, among cells with normal bundle orientation, half are patch-related and half are not patch-related (112 vs. 129 cells) (Fisher’s Exact Test, \( p = 1.05 \times 10^{-12}, n = 411 \) cells) (Fig. 7C).

Orientation defects are not limited to cells that misexpress Vangl2, which indicates that misleading polarity information must be transmitted from one cell to another. In conclusion, Vangl2 overexpression cell clusters induce non-autonomous polarity disruptions in neighboring cells of the auditory epithelium.

We reasoned that the location of a disturbed cell relative to a Vangl2 overexpression patch could offer insight into the directionality of polarity transmission from cell to cell. At locations abneural to a patch (see Fig. 7A), many cells are misoriented (Fig. 7D). Specifically, two-thirds of the cells located abneurally have an orientation defect (52 out of 78 cells). This contrasts with the neural side of the patch (see Fig. 7A), where most of the adjacent cells have a normal orientation (42 out of 56 cells) (Fig. 7D). The correlation between misorientation and the cell’s location, abneural versus neural, is highly significant (Fisher’s Exact Test, \( p = 1.62 \times 10^{-6}, n = 134 \)). Cells located to the sides of the misexpression patch are equally as often disturbed or normal (28 versus 23, \( n = 51 \) cells) (Fig. 7D). In summary, there is directionality in the local propagation of inaccurate polarity information caused by ectopic Vangl2 expression, which leads to non-cell-autonomous effects of hair cell misalignment in sensory epithelia.

Vangl2 overexpression locally inhibits bundle reorientation

We next asked whether RCAS/cLtap misexpression could overwrite the regional reorientation that is characteristic of the avian BP (reviewed by Gleich and Manley, 2000). Although hardly noticeable in the base (proximal), reorientation becomes obvious in the broad central area towards the distal BP. Several rows of hair cells closest to the abneural and neural edges are aligned parallel to the edge with an angle of ~180°, while centrally located hair cells in the distal two-thirds of the organ tend to shift their bundle orientation to face towards the apex. This increases the orientation angles up to 270°. Thus, average hair cell orientation angle differs systematically with proximal–distal and epithelium width location in normal ears (Tilney et al., 1987).

The BPs used in the above analysis of non-cell-autonomous effects were both sampled from locations that should reorient towards the apex. In addition to the sample shown in Fig. 6, the other sample area is shown in Figure S5. The precise sample locations for these specimens can be found in the figure legends; they range from 55% to 67% length (at 66% width) and are among the most apical samples in our data set. In both examples, the shift of unaffected cells (in blue) towards the apex is obvious. However, an overwhelming number (156 out of 170) of the affected cells are misoriented in one direction relative to the normal expected orientation of unaffected (or control) cells in this region of the BP. That is, 92% of the misoriented bundles are angled towards the abneural edge, albeit with a large amount of variation. This is the direction that all the bundles are expected to face prior to the reorientation phase and would manifest as smaller bundle angles when compared to cells that have reoriented apically. This trend can be seen quantitatively in orientation angle histograms when plotted in sample groups sorted for length location on the BP (Fig. 8, based on all 60 sample areas). Specifically, the histograms derived from length positions 30–39% and >39% both show an overrepresentation of smaller bundle angles in experimental ears as compared to

![Fig. 7](image-url)
controls. In contrast, both the control and the experimental histograms derived from length positions <30%, where bundles do not reorient or where reorientation is subtle, are symmetrically distributed about the same mean, with only the variance being obviously different. Thus, it appears that Vangl2 overexpression also interferes with the reorientation phase for cells that were either directly infected or that were influenced non-cell autonomously by their infected neighbors.

However, a summary histogram (Fig. 5B) representing all quantified BP locations does not clearly show evidence of this shift towards smaller orientation angles in experimental ears, perhaps because so many of the samples are from regions with little to no reorientation in normal specimens. Also, a high variance of hair cell orientation angles in RCAS/cLtap BPs and some variation in the percentage of uninfected cells per sample may both contribute to an incomplete penetrance of a "reorientation phenotype". Of course, all cells will influence the overall averages. In fact, there remains a progressive shift upward in orientation angles from base to apex, even in RCAS/cLtap-infected ears. The tendency towards larger angles with more distal (apical) positions in both virus-injected ears and controls can be seen by plotting location vs. mean orientation angle and performing linear regression on both data sets (Fig. 9); the correlation coefficient (control, 0.67 and RCAS/cLtap, 0.61) and slopes (control, 0.53 and RCAS/cLtap, 0.48) are quite similar. Thus, we conclude that reorientation is evident in the infected ears, possible due to the relatively large contribution of the uninfected cells.

**Discussion**

**Persistence of intracellular polarity and rough global alignment in Vangl2-infected sensory organs**

Our data show that intracellular polarity of hair cells appears intact following infection with RCAS/cLtap, which is similar to the results from Ltap mice (Lp) that carry a semi-dominant mutation of Vangl2 (Montcouquiol et al., 2003). Both the overall bundle morphology and its asymmetric placement on the cell surface are normal in infected hair cells, regardless of whether or not the cell has a defective orientation. This observation is in line with the growing evidence that disruption of Vangl2 expression does not interfere with ciliogenesis but instead affects downstream cell orientation and positioning of cilia such as the kinocilium (Borovina et al., 2010; Guirao et al., 2010; Song et al., 2010).

Misexpression of Vangl2 leads to cochlear hair cell orientation defects either by excessive expression as shown in this study or by dominant negative proteins (Montcouquiol et al., 2003; Song et al., 2010). Thus, any change to the delicate stoichiometry of a PCP complex disrupts function. Hence an experimental design using overexpression is expected to be very efficient compared to an incomplete gene knock down, especially given that recent data suggest redundancy of Vangl1 and Vangl2 in PCP of the mouse cochlea (Song et al., 2010). Excess Vangl2 interferes with restriction of the protein to the membrane; instead Vangl2 accumulates in the

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![Fig. 8](image-url) Comparisons of control (pink) versus RCAS/cLtap (blue) hair cell orientations sorted by position along the length of the BP. Frequency histograms are displayed from left to right according to the position of the sample regions along the BP from base to apex, respectively. The percent distance from the base is given as length (L). The histograms of hair cell orientations for the base (<30% L) are derived from 1030 cells (n = 12 BPs) of control ears and 868 cells (n = 14 BPs) of RCAS/cLtap experimental ears. The histograms for the mid-base (30–39% L) are based on 955 cells (n = 8 BPs) for controls and 483 cells (n = 5 BPs) for experimentals. The histograms for the mid-apex (>39% L) are based on 1331 cells (n = 10 BPs) for controls and 1467 cells (n = 11 BPs) for experimentals.

![Fig. 9](image-url) Correlation between average hair cell orientation and length position of the analysis area on the basilar papilla (BP). Control (pink) and RCAS/cLtap (blue) data points are mean hair cell orientation angles for 60 analysis areas along the BP length of 10 embryos.
cytoplasm as shown here (Fig. 4F) and with Vangl2BP-GFP fusion protein in MDCK cells (Montcouquiol et al., 2006).

In RCS/ClTap auditory epithelia, the initial broad alignment takes place as evidenced by the fact that hair cells are not randomly oriented. Instead, orientation angles fail to refine, leading to a larger variance in bundle angles. The disruption of cell orientation can be transferred to neighboring cells in a non-autonomous fashion, as discussed below. Even in epithelia with obvious failures in refinement of bundle orientation, the regional shift of uninformed hair cells (i.e., reorientation) that is characteristic of the BP, takes place. However, many infected cells and their nearest abneural neighbors do not reorient, and they also do not refine their bundle orientations. We conclude that Vangl2 misexpression interferes with both hair cell reorientation and the propagation of orientation information during the refinement phase of bundle orientation.

Prospectively, the bird's auditory epithelium in particular may become an important model to understand the mechanisms underlying developmental changes in cell polarity during development, due to its pronounced phase of reorientation that is not observed in mammalian cochlea. An analogous reorientation process occurs in epithelium of the fly wing. There, reorientation was recently shown to be associated with polarized axes of cell division, orientated cellular flow leading to elongation and narrowing of the wing primordium, and rearrangements of neighboring cells that is accompanied by a biased orientation of new vs. stable cell boundaries marked by Vangl2 expression (Aigouy et al., 2010). While hair cell reorientation in the avian BP takes place after the cessation of progenitor cell divisions (Katayama and Corwin, 1989), like the fly it overlaps temporally with a significant elongation of the organ (Tilney et al., 1986) and is preceded by a period of significant cell–cell rearrangements between hair cells and supporting cells from E9 to E12 (Goodyear and Richardson, 1997). Our data show that reoriented regions of the BP display Vangl2-positive cell boundaries that likewise are reoriented along the new polarity axis.

Directional propagation of PCP

In Drosophila, disruption of the subcellular localization of specific core PCP proteins, either through gain-of-function or loss-of-function, not only alters polarity of the mutant cells, but also propagates to adjacent cells such that wild-type cells can also become disoriented (Lawrence et al., 2004; Vinson and Adler, 1987). Specifically, clones misexpressing Vang cause disorientations on one side of a clone, while clones misexpressing Frizzled protein for example cause disorientations on the opposite side of the clone. These analyses are possible because of the ability to generate mosaic flies in which small clones of mutant cells are generated in a wild-type background. The confirmation of non-cell-autonomous polarity defects, along with a clear directionality in the propagation of those defects on one side of the clone, has been instrumental in modeling the underlying molecular mechanisms by which PCP is mediated in Drosophila (Lawrence et al., 2007). In vertebrates, Vangl2 acts non-cell-autonomously in several different situations, including the process of neurulation in zebrafish (Ciruna et al., 2006), the polarization of mouse hair follicles (Devenport and Fuchs, 2008), and the anterior–posterior orientation bias of cilia in Xenopus skin cells (Mitchell et al., 2009). In each of these examples, changes in the level of functional Vangl2 protein produce non-cell-autonomous effects on nearby wild-type cells. Moreover, Mitchell and colleagues (2009) elegantly used mosaic analysis of Vangl2– or Fzd3–misexpressing cells to provide evidence for inherent directionality in the propagation of defects in ciliary orientation of Xenopus skin cells.

We have used virus-mediated gene transfer to create analogous mosaics within the auditory sensory epithelium. In this experimental design, cells overexpressing Vangl2 confront wild-type cells that have endogenous levels of Vangl2. Although the viral-infected cell clusters are somewhat less cohesive than those that can be created in flies using clonal recombinant methods, it is indeed possible to observe a directional bias in the misorientation of wild-type cochlear hair cells. Our data show that uninfected cells on the abneural side of the cluster have a higher probability of showing orientation defects than cells on the neural side of the cluster. Cells on the remaining two sides of the cluster (proximal and distal) are intermediate in their level of disturbance. Molecular mechanisms that control directional polarity propagation and integrate different core PCP components, including Vangl2, remain to be explored in detail. By analogy to the fly, we favor the idea of a feedback system with attractive interactions at cell junctions across cell borders (e.g., between Vangl2 and Fzd3/6) and repulsive interactions within cells (e.g., between Pk and Dvl) that maintain intracellular polarity. To date there is limited information about the subcellular distribution of the full set of core PCP proteins in the BP. We attempted to localize Fzd3 using an antibody derived against a peptide from the mouse Fzd3 that is relatively well-conserved with the chicken, but no obvious asymmetries were observed in the apical epithelium of the BP. The absence of such data presents difficulties in generating an unambiguous molecular model for polarity propagation that compares to the fruit fly model and fully incorporates the vertebrate PCP proteins that have been implicated in hair cell polarity, including Ptk7 (Lu et al., 2004), Scrib (Montcouquiol et al., 2003), Frizzleds, Dishevelled, Prickle, Celsr (Wang and Nathans, 2007) and Cthrc1 (Yamamoto et al., 2008). Most problematic is that the orthologs of two proteins described as being expressed on opposite sides of the cell in flies (Fz and Vang) are reportedly localized to the same side of the hair cell in the mouse cochlea (Montcouquiol et al., 2006; Wang et al., 2006). Wang and Nathans (2007) argue, based on fly data, that PCP protein localization may not be primarily instrumental for planar polarity, but more a consequence of its effects. This idea is more parsimonious with the apparent uncoupling of the bundle orientations and PCP protein asymmetry observed at the striola in mouse vestibular organs, where hair bundles abruptly reverse direction despite a uniform directionality of PCP proteins (Deans et al., 2007). Furthermore, unlike in flies, mutation of Vangl2 does not change the levels of Fzd3 and Fzd6 proteins in mouse inner ears, although the proper membrane localization of Frizzleds is abolished (Wang et al., 2006). Nonetheless, our novel observation of the directional propagation of PCP information in the vertebrate sensory system is provocative in implicating some common mechanisms across evolution, at least with respect to Vang orthologs. Notably, though, our evidence that supporting cells, but not hair cells, are the core PCP protein expressors while hair bundle asymmetries are being established (Fig. 3A, B) highlights an important difference between PCP mechanisms acting in the auditory organ of vertebrates versus Drosophila mechanosensory cells, where there is a uniform array of cells all behaving similarly.

Evidence for the presence of a global polarity cue in the chicken BP

The misexpression of Vangl2 does not completely negate the inherent polarity bias: bundles are neither inverted nor randomized in orientation. In fact, the quadrant of bundle angles representing neural orientation was never recorded in Vangl2-infected ears. This is strikingly different from other model systems. In the fly, gain-of-function for Vang completely reverses the polarity of wild-type cells immediately adjacent to an overexpressing clone and cells within the clones are generally disarranged (Lawrence et al., 2004). Likewise, wild-type ciliated cells located immediately anterior to a clone of Vangl2-overexpressing cells in Xenopus skin show nearly complete reversal of their normal orientation; this is a dominating non-cell-autonomous effect (Mitchell et al., 2009). In both model systems, polarity reversal occurs on the opposite side of clones lacking Vang/ Vangl2. Parenthetically, the cell-autonomous effects of Vangl2 overexpression that might be expected to randomize cell polarity were not
examined in the Xenopus model. Among cochlear hair cells, the stereociliary bundles in lp (Vangl2 mutant) mice are randomized only for the third-row outer hair cells (Montcouquiol et al., 2003). Notably, the majority of lp hair bundles in the other three rows remain biased towards the abneural direction, a result not dissimilar to Vangl2 overexpression in chicken. Domineering non-cell-autonomous bundle reversal cannot be explored in lp mice, since all cells carry the mutant allele.

A potential limitation of the retroviral approach is variability in the onset of infection and hence protein overexpression. Cells in our sample regions (which avoided the neural, abneural and distal extremes) are likely to express high levels of Vangl2 by E4–E8, based on an inherent delay between integration and robust protein translation and on the timing of cell-cycle withdrawal in the centralmost BP (Katayama and Corwin, 1989). This offers an interval of several days between the onset of enhanced Vangl2 levels and the initiation of bundle differentiation.

Setting aside possible technical limitations in the retroviral approach, our data in the chicken imply the persistence of an initial global polarity cue along the neural–abneural axis that can influence hair bundles either prior to or, in parallel with, the Vangl2-mediated transmission of PCP. The nature of this global cue is likely to differ from that in Drosophila, primarily due to the lack of evidence that Wingless is required to instruct PCP in flies. In Drosophila, there appear to be two independently-acting PCP pathways. One involves gradients of Four-jointed and Dachsous, while the other is thought to utilize a Frizzled concentration gradient that feeds into the Starry night system with Vang protein involvement. Both pathways may be driven in turn by morphogen(s) (Lawrence et al., 2007).

There is gathering evidence that the global polarity cue(s) in vertebrate sensory organs may involve Wnts as extracellular ligands binding to Frizzled receptors and perhaps directly to Vangl2. While heterozygous Wnt5a mice show few hair cell orientation defects in the organ of Corti, double-heterozygous Wnt5a/Ltap (Vangl2) mice display misorientation of third-row outer hair cells; these data reveal that Wnt5a and Vangl2 can interact genetically (Qian et al., 2007). In the same study, hair bundles are disrupted in cultured hearing organs by exogenous application of the Wnt inhibitor, Sfrp3 (Fzb), an effect that can be abrogated by simultaneous treatment with Wnt5a protein. On the other hand, incubation with Wnt5a protein alone has no effect on bundle orientation in mice, a finding that is consistent with our results using RCAS/Wnt5a in chicken embryos. Introduction of exogenous Wnt7a to a cultured organ of Corti can disrupt hair cell orientation in mice (Dabdoub et al., 2003). Thus, selectivity for specific Wnt ligands appears to be important, although endogenous ligands that have an instructive role in bundle polarity have yet to be identified and, if present, may vary between species. Wnt involvement in chicken hair cell orientation is further implicated by blocking the activity of JNK, a downstream component of the non-canonical/PCP Wnt signaling pathway (Warchol and Montcouquiol, 2010). Wnt sources are indeed present in the chicken auditory organ anlage as both neural and abneural tissues abutting the organ primordium express different Wnt transcripts in the appropriate time window (Sienknecht and Fekete, 2008). Promising Wnt candidates for mediating polarity in the chicken BP are Wnt11 and Wnt9a (from the neural side) and Wnt5b (from the abneural side). Some of these Wnts have already been implicated in PCP in the directional movement of cells during gastrulation, a process called convergent-extension. Wnt11 genetically interacts with Vangl2 during zebrafish gastrulation (Vervenne et al., 2008). Triple mutant zebrafish for Wnt11/Wnts5/Wnt4 have a PCP defect and they fail to localize Pk protein (Ciruna et al., 2006). Gain of Pk1 during zebrafish convergent-extension movements is thought to down-regulate Dvl protein levels, thus inhibiting the Fzd7-dependent membrane localization of Dvl (Carreira-Barbosa et al., 2003). Wnt responsiveness requires receptor proteins, two of which (Fzd7 and Fzd10) are expressed in the embryonic BP in a graded fashion across the axis of planar polarity, being higher abneurally (Sienknecht and Fekete, 2008).

Fz protein recruitment to the adherens junction zone during PCP signaling has been described for Drosophila via a potential mechanism where an array of microtubules with a bias in orientation is operational. This orientation bias is controlled by Dachsous and Fat, membrane proteins that can act upstream of PCP (Shimada et al., 2006). Whether this in turn directly leads to binding and polarized membrane accumulation of core PCP proteins such as Vangl2 and Fzd3/6, or whether this indirectly induces a microtubule-based protein transport mechanism, remains unanswered. The involvement of the cytoskeleton is evolutionarily conserved, since depolymerization of microtubules during zebrafish gastrulation blocks asymmetric (anterior) accumulation of Pk. The link between the cytoskeleton and Wnt/PCP signaling appears to be reciprocal in zebrafish: a bias of the microtubule organizing center towards the opposite (posterior) side of the cells is lost when two Wnt/PCP components, glypicican4 and Dvl, are perturbed (Sepich et al., 2011).

Correlations between ciliary proteins and PCP of the inner ear (reviewed by Jones and Chen, 2007) raise the intriguing possibility that the kinocilium may serve as the sensing “antenna” for detection of a diffusible extracellular gradient. For example, the ciliary protein inversin both inhibits canonical Wnt signaling and modulates the noncanonical Wnt/PCP pathway during convergent-extension movements in Xenopus gastrulae (Simons et al., 2005). In summary, Wnts that have been implicated in PCP in other systems may be diffusing across the chicken BP as a gradient to provide directional information. Although it is notable that removal of a potential signaling center, the striola region of chicken utricle explants, does not result in PCP defects (Warchol and Montcouquiol, 2010). Interestingly, however, recent analyses of Drosophila wing development suggest that no long-range signaling is necessary to establish and maintain global polarity and that a coordinating role of the F1/Ds pathway in the fly is consistent even when the polarity vectors change during development as reorientation takes place (Aigouy et al., 2010).

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