PCP and Wnt pathway components act in parallel during zebrafish mechanosensory hair cell orientation

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Planar cell polarity (PCP) plays crucial roles in developmental processes such as gastrulation, neural tube closure and hearing. Wnt pathway mutants are often classified as PCP mutants due to similarities between their phenotypes. Here, we show that in the zebrafish lateral line, disruptions of the PCP and Wnt pathways have differential effects on hair cell orientations. While mutations in the PCP genes vangl2 and scrib cause random orientations of hair cells, mutations in wnt11f1, gpc4 and fzd7a/b induce hair cells to adopt a concentric pattern. This concentric pattern is not caused by defects in PCP but is due to misaligned support cells. The molecular basis of the support cell defect is unknown but we demonstrate that the PCP and Wnt pathways work in parallel to establish proper hair cell orientation. Consequently, hair cell orientation defects are not solely explained by defects in PCP signaling, and some hair cell phenotypes warrant re-evaluation.
Cell polarity is crucial for the function of many tissues and its establishment during development has fascinated many generations of biologists. In addition to the well-studied apico-basal polarity of cells, cells are also coordinately aligned in the plane of a tissue’s axis, termed planar cell polarity (PCP). PCP relies on the asymmetric distribution of core PCP components, such as Van Gogh/Vangl, Frizzled, Disheveled, Prickle/Spiny-Legs, and Fmi/Celsr and Dielgo1–3. This pathway was discovered in insects4–8 and subsequently also identified in vertebrates9–11. PCP is required during key developmental processes that shape the embryo, such as gastrulation and neural tube closure1–3, however, how PCP is initiated is poorly understood. One proposed mechanism is that Wnt gradients act as instructive morphogens that initiate the molecular asymmetry of PCP components12–17. However, in some contexts Wnt ligands are required but not instructive (permissive) to drive PCP-dependent processes18–20. In this study, we set out to investigate the role of Wnt signaling in polarizing sensory hair cells.

The vertebrate inner ear is a classical model to study the function of the PCP pathway, as stereocilia bundle coordination is very sensitive to changes in PCP21,22. Wnt gradients have been implicated in establishing PCP in the ear23,24 but due to the inaccessibility of the ear, the study of the function of the PCP and Wnt signaling pathways in coordinating hair cell alignment is challenging to investigate.

A more experimentally accessible model to study hair cell orientation is the sensory lateral line system of aquatic vertebrates that detects water movements across the body of the animal25,26. Because of its superficial location in the skin, the lateral line is amenable to experimental manipulations and live imaging27. The lateral line system consists of volcano-shaped sensory organs (neuromasts) that are composed of mantle cells on the outside and support cells and mechanosensory hair cells in the center (Fig. 1a, b25). The mechanosensory hair cells are homologous to the ones found in the inner ear28,29. The sensory organs are derived from several neurogenic, cephalic placodes/homologous to the ones found in the inner ear28,29. The sensory organs are derived from several neurogenic, cephalic placodes/primordia that either migrate into the trunk or into the head30. As they migrate, primordia periodically deposit clusters of cells that differentiate into sensory organs31–33. PrimordiumI (primI) and primordiumII (primII) both migrate into the trunk but arise from different placodes (primary placode and D0 placode, respectively). The D0 placode also gives rise to a third primordium that migrates onto the dorsal side of the trunk, called primIII34,35.

Hair cells possess short actin-rich stereocilia adjacent to a tubulin-rich, long kinocilium. Each hair cell is planar polarized with the long microtubule-based kinocilium localized to one pole of the cuticular plate (Fig. 1b). Within a sensory organ, hair cells arise in pairs with their kinocilia pointing toward each other but are aligned along a common axis (Fig. 1c). The positioning of the kinocilium along this axis is controlled by Notch signaling/Emx2 and its loss causes all hair cells within a neuromast to point into the same direction35,36. PrimI and primII-derived hair cells show different axial polarities, allowing the animal to sense water flow in different directions (Fig. 1c, 37). In primI-derived trunk neuromasts, hair cells are planar polarized along the anterioposterior (A-P) axis and hair cells in primII-derived neuromasts are aligned along the dorsoventral (D-V) axis. The differential hair cell orientation has been correlated with the different directions of primI and primII migration, however, no underlying mechanism has been identified35,34,37. At the single hair cell level, previous reports have suggested that PCP regulates hair cell progenitor orientation by controlling cell division angles and cell rearrangements38,39. Yet the molecular code for how different neuromast axial orientations and individual hair cell orientations are coordinated remains ill understood.

Here, we show that loss of PCP and Wnt pathway genes (wnt11 (wnt11f1)) and gpc4 and fz7a/7b) have different consequences on hair cell orientations. While mutations in the PCP genes vangl2 and scrib cause disorganized hair cell orientations in all neuromasts, mutations in the Wnt pathway genes wnt11 (wnt11f1), gpc4 and fz7a/7b show a striking concentric pattern of hair cell orientation in only primII neuromasts. As neither the core PCP component Vangl2, nor Notch/Emx2 signaling are affected in Wnt pathway mutants we conclude that the Wnt pathway acts in parallel to these pathways. In addition, the concentric hair cell phenotype in Wnt pathway mutants is caused by the disruption of coordinated organization of the surrounding support cells, rather than by affecting the axis of polarity or kinocilium positioning in individual hair cells.

The expression patterns of Wnt pathway genes suggest that the Wnt pathway acts very early in lateral line development. Thus, Wnt signaling does not instruct PCP, but acts to coordinate support cell organization during the formation and migration of the primordium before the appearance of hair cells. The molecular mechanisms by which Wnt signaling coordinates support cell orientation remains to be elucidated. Overall, our findings demonstrate that hair cell orientation defects cannot solely be attributed to defects in the PCP pathway and that some phenotypes formerly characterized as PCP defects need to be re-evaluated.

Results

Wnt and PCP genes cause different hair cell orientation phenotypes. During a large in situ screen, we unexpectedly observed asymmetric expression of wnt11 (wnt11f1), the ortholog of mammalian WNT1140, wnt11 (wnt11f1) is expressed along the anterior edge of only primI-derived neuromasts, but is absent from primII-derived neuromasts (Fig. 1d, Supplementary Fig. 1). Since Wnt ligands can instruct planar polarization of cells10,16,17,41–44, we hypothesized that wnt11 (wnt11f1) establishes hair cell orientation by directing PCP in primI-derived neuromasts. We measured hair cell orientation in the cuticular plate using Phalloidin, which labels actin-rich stereocilia but not the tubulin-rich kinocilium (Fig. 1b). We used the kinocilium position to determine the axis of polarity of each hair cell. Phalloidin stainings of sibling primI-derived neuromasts show that hair cells possess a significant orientation bias parallel to the A-P axis based on the angles with respect to the horizontal in rose diagrams (Fig. 1e). In contrast, primII-derived neuromasts show an orientation bias along the D-V axis (Fig. 1k). Furthermore, neighboring hair cells in both primordia show coordinated polarities (Supplementary Fig. 1c, i). Unexpectedly, zygotic and maternal zygotic (MZ) mutations in wnt11 (wnt11f1) do not affect hair cell orientation in primI-derived neuromasts in which wnt11 (wnt11f1) is expressed (Fig. 1f; Supplementary Fig. 1d), but disrupt the hair cell orientation in primII-derived neuromasts (Fig. 1f; Supplementary Fig. 1j). Even though zygotic wnt11 (wnt11f1) mutants also show the phenotype, we from here on used MZwnt11 (wnt11f1) larvae to increase the number of mutant fish for our studies.

gpc4 (glypican4), a heparan sulfate proteoglycan (HSPG) and fz7a (a frizzled-class receptor) interact with Wnt ligands during convergent extension (CE), and we tested whether mutations in these genes also cause hair cell polarity defects48–51. Indeed, gpc4 and MZfz7a/7b mutants show the same concentric hair cell phenotype as MZwnt11 (wnt11f1) mutants suggesting they act in the same pathway (Fig. 1g, m; and h, n; Supplementary Fig. 1e, f, k, l). This interpretation is supported by the finding that wnt11 (wnt11f1);gpc4 double mutants possess the same phenotype as the
single mutants (Supplementary Fig. 1o). gpc4 and fzd7a/7b also interact with wnt11f2 (formerly called wnt11/silberblick,40) during CE18,50,51. We, therefore, wondered if wnt11f2 mutants also possess hair cell defects and if wnt11f2 possibly interacts with wnt11 (wnt11f1). However, wnt11f2 mutants have normal hair cell orientations, as do wnt11 (wnt11f1);wnt11f2 double heterozygous animals, indicating that these two paralogs do not interact (Supplementary Fig. 1p).

Since gpc4 and MZfzd7a/7b mutants have been described as PCP signaling mutants in other contexts45,46,52,53, we compared the phenotypes of MZwnt11 (wnt11f1), gpc4 and MZfzd7a/7b mutants to the hair cell phenotype of the PCP mutants vangl2 and scribble (scrib). A mutation in the core PCP gene vangl2 disrupts hair cell orientation in both primI and primII-derived neuromasts38,39 (Fig. 1i, o; Supplementary Fig. 1g, m). Scribble1 interacts with the PCP pathway in the mouse cochlea and its loss...
Mutations in the Wnt and PCP pathways affect the hair cell alignment in the lateral line. **a** Confocal image of a 5 dpf larva Tg(cldnb:lynGFP); Tg(gata4); Tg(cldnb:H2A-mCherry) larva. **b** Schematic lateral view of a 5 dpf neuromast showing the different cell types. **c** Diagram of a 5 dpf larva showing the different orientations of priml and primll-derived hair cells. **d** In situ hybridization of mZwnt11 (wnt11ff1) in priml and primll-derived 5 dpf neuromasts. **e**–**j** Phalloidin stainings show hair cell orientations in priml-derived neuromasts of wild type (**e**), Wnt pathway mutants (**f**–**h**) and PCP mutants (**i**–**j**). Fisher’s Exact Test p-value for vangl2 priml = 7.33 × 10−28, mZscrib priml = 1.41 × 10−17. Individual hair cell orientation is depicted for each of the conditions tested. Black arrows denote disruption of the wild-type orientation. The Rose diagrams show the hair cell orientation distribution with respect to the longitudinal axis of the animal (horizontal). **WT n = 194 hair cells, MZwnt11 (wnt11ff1) n = 353, gpc4 n = 222, MZfzd7a/7b n = 74, vangl2 n = 226, MZscrib n = 353.** Bottom right depicts the Fisher’s exact test comparison with respect to the wild type for each condition. Top right shows the binomial test for each condition. **k**–**p** Phalloidin staining shows hair cell orientation in priml-derived neuromasts of wild type (**k**), Wnt pathway mutants (**l**–**n**) and primll-derived neuromasts (**o**–**p**). This difference in phenotype suggests that the Wnt and PCP signaling pathways control hair cell orientation in parallel, rather than through a common pathway. However, Wnt and PCP pathways have been described to interact genetically in the establishment of hair cell orientation in the inner ear. To assess a possible genetic interaction between vangl2 and mZwnt11 (wnt11ff1) in lateral line neuromasts we generated double homozygous vangl2 and MZwnt11 (wnt11ff1) larvae. In double homozygous fish, priml- and primll-derived neuromasts show random hair cell orientation (**l**–**n**) and two different orientations (**o**–**p**). Since loss of vangl2 disrupts the concentric phenotype, we hypothesized that PCP signaling might be correctly established in hair cells of the Wnt pathway mutants. To test whether one of the landmarks of PCP signaling, asymmetric distribution of Vangl23,4, is disrupted in hair cells of Wnt pathway mutants we performed anti-Vangl2 antibody stainings in 5 dpf neuromasts (**q**). In wild type neuromasts, Vangl2 is asymmetrically enriched in approximately 90% of hair cells. Vangl2 is localized to the posterior side of hair cells in priml-derived neuromasts (**r**). However, as hair cells in a wild type mutant possess two different orientations (**s**), Vangl2 staining is not detected in vangl2 mutants (**t**–**v**). MZscrib mutant fish show reduced signal and only 26.3% of hair cells with asymmetric Vangl2 in priml and primll-derived neuromasts (**w**–**y**). In contrast to the PCP mutants, MZwnt11 (wnt11ff1) mutants show asymmetric distribution of Vangl2 in hair cells of both priml and primll-derived neuromasts showing that PCP signaling is not affected (**z**–**ab**).
localization, we generated a hair cell-specific promoter-driven GFP-Vangl2 
\text{Tg(myo6b:GFP-XVangl2). We then calculated GFP fluorescence enrichment around the circumference (−180° to 180°) of the cuticular plate at the base of the stereo- and kinocilia with 0° on the x-axis being the point of maximum intensity (Fig. 3j–o). In wild-type larvae mosaic expression of fluorescent Vangl2 is asymmetrically enriched in hair cells of primI and primII-derived neuromasts (Fig. 3j–l, p). In MZwnt11 (wnt11f1) mutants, GFP-Vangl2 is asymmetrically localized in primI and primII-derived neuromasts like in wild type hair cells (Fig. 3m–p). These results indicate that in contrast to the PCP mutant vangl2, individual hair cell GFP is normal in Wnt pathway mutants. These findings suggest that the concentric arrangement of hair cells in Wnt pathway mutants is caused by a PCP-independent mechanism.

The Wnt and PCP pathways have different temporal requirements. The differences in the hair cell orientation phenotypes of Wnt and PCP pathway mutants suggest that they do not act in
the same pathway. To identify further differences in their phenotypes we investigated PCP-dependent cell behaviors of hair cell progenitors in vivo. The two daughters of a hair cell progenitor often change their position with respect to each other before differentiating\(^38,39\) (Supplementary Movie 1). These cell rearrangements and their duration depend on functional PCP signaling during development and regeneration. In vangl2 mutants the hair cell rearrangements do not lead to complete reversal of positions, they sometimes occur multiple times and their duration is prolonged\(^39\). In contrast, time-lapse analyses of the behavior of developing hair cell progenitors in primII-derived neuromasts of MZwnt11 (wnt11f1) mutants reveals no significant
Fig. 3 Vangl2 asymmetry is not affected in hair cells of MZwnt11 (wnt11f1) mutants. a-d Double β-Il-Spectrin (labeling the cuticular plates) and Vangl2 immunodetection in primI neuromasts of 5 dpf wild type (a), MZscrib mutants (b), scrib siblings (c) and MZwnt11 (wnt11f1) mutants (d). e-h Double β-Il-Spectrin (labeling the cuticular plates) and Vangl2 immunodetection in primII neuromasts of 5 dpf wild type (e), MZscrib mutants (f), scrib siblings (g) and MZwnt11 (wnt11f1) mutants (h). i Quantification of the percentage of hair cells that show asymmetric Vangl2 for each of the conditions in both primordia-derived neuromasts. j-n Clonal localization of GFP-XVangl2 in hair cells of neuromasts that show primI and primII polarity in wild type (j, k) and MZwnt11 (wnt11f1) mutants (m, n). Neomycin62 by CRISPR/Cas9-mediated genome editing (Supplementary Fig. 5c, d). While the uninjected wild-type siblings MZscrib mutants (Fig. 5g) and gpc4, primI-derived support cells are horizontally aligned (Fig. 5i), whereas support cells in primII-derived neuromasts do not possess an evident coordinated vertical organization (Fig. 5j; Supplementary Fig. 5b). To investigate a possible genetic interaction between PCP and Wnt pathway genes during the establishment of support cell organization, we asked whether the support cell phenotype in Wnt pathway mutants would disrupt the support cell alignment in PCP mutants. Double vangl2,MZwnt11 (wnt11f1) homozygous mutants indeed show a loss of alignment in primII-derived neuromasts and normal, horizontal alignment of support cells in primI neuromasts as characteristic for single MZwnt11 (wnt11f1) mutants (Fig. 5k, l). This result shows that the wnt11 (wnt11f1) phenotype in support cells is epistatic and disrupts normal support cell alignment in PCP mutants.

Wnt pathway genes coordinate support cell organization. As the hair cell defect arises over time in MZwnt11 (wnt11f1) mutants we wondered if the hair cell phenotype could be secondary to defects in neighboring support cells. Support cells surround and sit underneath developing hair cells and serve as hair cell progenitors. Interestingly, support cells are aligned along the A-P and D-V axes in primI and primII-derived neuromasts, respectively, raising the possibility that the Wnt pathway genes might be acting on them (Fig. 5a, b).

To better visualize any defects in support cell organization we removed hair cells by soaking the larvae in the antibiotic neomycin and calculated their orientation with respect to the horizontal (Supplementary Fig. 5a). In wild-type larvae, support cells show a coordinated, elongated alignment along the horizontal plane (A-P axis) in primI-derived neuromasts (Fig. 5c) and vertical plane (D-V axis) in primII-derived neuromasts (Fig. 5d). These cell alignments demonstrate that hair cell ablation does not disrupt intrinsic support cell alignment. Unexpectedly, we observed that in vangl2 mutants primI-derived support cells are normally aligned along the horizontal axis, even though their hair cell orientation is randomized (Fig. 5e). vangl2 mutant support cells in primII-derived neuromast still align, but offset by 90° horizontally (Fig. 5f), a finding we currently cannot explain. Thus, support cells in primII-derived neuromasts of vangl2 mutants show a cell orientation defect, but not randomization. This suggests the vangl2 might play some not understood role in only primII-derived support cell orientation. However, the MZscrib PCP mutant shows wild type support cell organization in both primI (Fig. 5g) and primII-derived neuromasts (Fig. 5h). These results imply that functional PCP might not be required for coordinated support cell alignment but that PCP genes act mostly in hair cells. On the other hand, in the Wnt pathway mutants MZwnt11 (wnt11f1) and gpc4, primI-derived support cells are horizontally aligned (Fig. 5i), whereas support cells in primII-derived neuromasts do not possess an evident coordinated vertical organization (Fig. 5j; Supplementary Fig. 5b). To investigate a possible genetic interaction between PCP and Wnt pathway genes during the establishment of support cell organization, we asked whether the support cell phenotype in Wnt pathway mutants would disrupt the support cell alignment in PCP mutants. Double vangl2,MZwnt11 (wnt11f1) homozygous mutants indeed show a loss of alignment in primII-derived neuromasts and normal, horizontal alignment of support cells in primI neuromasts as characteristic for single MZwnt11 (wnt11f1) mutants (Fig. 5k, l).

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wild-type atoh1a CRISPRants show no hair cells (Fig. 5o, p). In atoh1a CRISPRants support cells are still normally aligned (Fig. 5o, p). Thus, support cell alignment is independently regulated from hair cells. Furthermore, MZwnt11 (wnt11f1) mutants injected with atoh1a CRISPR still show loss of support cell alignment in primII-derived neuromasts (Fig. 5q, r). Thus, the disorganization of support cell orientation observed in primII neuromasts of Wnt pathway genes is not a consequence of the hair cell misorientation. Altogether, these results indicate that, while vangl2 and scrib are not required for support cell alignment, the Wnt pathway genes are essential for the proper coordinated organization of support cells in primII-derived neuromasts.
Wnt pathway genes likely act in the premigratory primordium. Because wnt11 (wnt11f1) is not expressed in 5 dpf primII neuromasts, which show a phenotype in MZwnt11 (wnt11f1) mutants (Figs. 1 and 5), we hypothesized that wnt11 (wnt11f1) might be required earlier in lateral line development to establish hair cell orientation. We tested if Wnt pathway members are expressed in primII or in adjacent tissues during migration or placode specification stages. At 50 hpf, while primII is migrating and has not yet deposited its first neuromast, vangl2 is expressed in primII, and in primI and primII-derived neuromasts, suggesting that it is acting within lateral line cells (Fig. 6a). Likewise, the other PCP gene scrib is detected in the lateral line by in situ hybridization, agreeing with a previous RNA-Seq analysis (Fig. 6b). In contrast to vangl2, wnt11 (wnt11f1) mRNA is not detectable in either the migrating primordia, or primI-derived neuromasts (Fig. 6c). However, it is expressed in the underlying muscle along the angle with respect to the horizontal.
myoseptum20,65, suggesting that the muscle could signal to the neuromasts (Fig. 6d; Supplementary Fig. 6a). In contrast to the wnt11 (wnt11f1) ligand, the Wnt co-receptor gpc4 is expressed in the migrating primII and primI-and primII-derived neuromasts, even though its loss only affects primII-derived neuromasts (Fig. 6e, Fig. 1). The expression of the fzd7a and fzd7b receptors is more complex. Both are expressed in the lateral line, while fzd7a is expressed in primII, and primII-derived neuromasts (Fig. 6f). fzd7b is not expressed in primII but is expressed in primI-and primII-derived neuromasts (Fig. 6g). As only fzd7a is expressed in the migrating primII but only fzd7a/b7 double mutants affect primII-derived neuromasts, we hypothesize that Wnt signaling is already acting during premigratory stages.

Analysis of the expression pattern of the Wnt pathway genes at a time point in which primII is being specified (32 hpf) shows that wnt11 (wnt11f1) is expressed in a dispersed group of cells posterior to the eye immediately adjacent to the forming, gpc4-expressing D0 placode (Fig. 6h-j arrowhead). In addition, wnt11 (wnt11f1) is expressed in the lens, brain, ear and dorsal spinal cord. The D0 placode gives rise to primII, primID (that gives rise to the lens, brain, ear and dorsal spinal cord). The role of vangl2 in support cells is therefore still unresolved.

Overall, these results suggest that while PGC genes are expressed and required to establish proper hair cell planar polarization within all neuromasts, Wnt pathway genes act earlier in support cells during D0 placode formation, prior to the differentiation of hair cells. Support cells then secondarily influence hair cell orientations in 5 dpf primII neuromasts. Thus, the loss of Wnt signaling early in development has effects on organ formation several days later.

Discussion

PCP and Wnt pathway genes are involved in the establishment of hair cell orientation in vertebrates23,24,54–56,67–71. Because disruptions of both pathways cause hair cell arrangement defects, Wnt pathway mutants are often classified as PCP mutants. Wnt ligands play different roles in different PCP contexts. For example, Wnt ligands instruct PCP15–17,44 or act as gradients that control hair cell orientation in the inner ear23,24. Our results demonstrate that in the context of neuromasts, the Wnt pathway genes wnt11 (wnt11f1), gpc4 and fzd7a/7b do not affect PCP signaling as Vangl2 localization is normal in Wnt pathway mutants (Fig. 3). In addition, in MZwnt11 (wnt11f1);vangl2 double homozygous neuromasts, hair cell orientations are randomized as in vangl2 mutants, suggesting that PCP signaling is normal in MZwnt11 (wnt11f1) mutants (Fig. 2). Recent reports show that Emx2 and Notch signaling control stereocilia bundle orientation and that Vangl2 is normally localized in emx2 mutants suggesting that this pathway acts in parallel to the PCP pathway36,72. As emx2 affects hair cell polarity without affecting PCP signaling, we wondered if the Wnt pathway genes acted in concert with Emx2 or Notch signaling. However, the number of Emx2-expressing hair cells is normal in MZwnt11 (wnt11f1), even though they are not localized to one half of the neuromasts but are randomly distributed (Supplementary Fig. 7a–e).

Therefore, Wnt pathway genes are not acting in the same pathway with Notch and Emx2 signaling to regulate hair cell orientation. Instead, our data suggest that Wnt pathway genes act in emx2-negative support cells, whose misalignment causes hair cell orientation defects secondarily in the absence of Wnt signaling.

An unresolved question is why in Wnt pathway mutants the concentric hair cell phenotype arises sequentially as more cells are added. A concentric orientation might represent the most energy-efficient arrangement for hair cells that still possess functional PCP but that are surrounded by misaligned support cells. Our results stress the importance of studying all cell types in an organ when analyzing hair cell phenotypes in vertebrates. Therefore, gpc4 and MZfze7a/7b mutants previously characterized as PCP signaling mutants in other contexts should be re-evaluated as such.
How are the different orientations in primI and primII-derived neuromasts achieved? PCP pathway mutations disrupt hair cell orientations in all neuromasts. Thus, the mechanisms that control differential A-P and D-V orientations in primI and primII-derived neuromasts are either acting upstream or in parallel to PCP signaling. Lopez-Schier et al. proposed that the direction of the migrating primordia or the recently deposited neuromasts determines the axis of polarity. Neuromasts deposited by primII migrate ventrally after deposition, which has been correlated with instructing the axis of hair cell polarity with a 90° angle with respect to primI-derived neuromasts. However, the adjacent primI-derived interneuromast cells also undergo a ventral migration and form neuromasts with primI-polarity. Likewise, the D1 neuromast forms from cells left behind by primI and possesses primI polarity (Supplementary Fig. 6c), even though it undergoes dorsal migration after being formed. Therefore, the direction of migration is not an indicator of hair cell polarity. Our data suggest that support cell polarity in different primordia is set
**Fig. 6** Temporal dynamic expression of the PCP and Wnt pathway genes during formation and migration of primII. a–g In situ localization on 50 hpf wild-type fish of mRNA for **vangl2** (a), **scrib** (b), and **wnt11** (wnt11f1) (c), at the level of the primordium; (d), at the level of the underlying muscle, *gpc4* (e), *fzd7a* (f), *fzd7b* (g). The first neurastem derived from primII is outlined using black, while primII is outlined in white in (a–g). h In situ localization of **wnt11** (wnt11f1) mRNA in a 34 hpf wild-type embryo. i Magnification of the expression in the area posterior to the ear in (h). The ear is delimited by a dashed line. j In situ localization of **gpc4** mRNA in the area posterior to the ear at 32 hpf. Arrowhead indicates the putative localization of the D0 placode. k–n Still frames of the time lapse analysis of the formation of primII, primD, occipital prim, and D1 labeled in a Tg(cldnb:lynGFP) transgenic wild-type fish (Supplementary Video 5). Arrow in k indicates the original group of cells that gives rise to all three primordia. Arrowheads in l–n indicate the different primordia formed. Asterisk in m, n indicates the position of the D1 neurastem.* o, p Schematic cartoon of the proposed mechanism by which Wnt11 (Wnt11f1) signals to cells in the D0 placode (o) to establish support cell organization (p). q t-SNE plots and violin plots showing expression of *fzd7b* (q) and **vangl2** (r) in a 5 dpf neurastem during homeostasis. The Wilcox *p*-value for *q* = 8.45 *×* 10−32 in support cells and mantle cells versus hair cells, while in r the expression pattern was too sparse to generate meaningful statistics. HC’s hair cells, SC’s support cells, MC’s mantle cells. ** denotes statistical significance. Scale bar in a equals 10 μm, h equals 50 μm, i equals 25 μm, k equals 50 μm up as they are formed, and which instructs hair cell polarity in later forming neurastems.

Wnt pathway genes are only required for support cell organization in primII- and not primII-derived neuromasts (Fig. 5) raising the question of how primII-derived neurastems are polarized. Support cells in primII neurastems could be organized by a mechanism that involves tissue tension as described in the skin of mouse74, ciliated epithelia of Xenopus75,76 and in Drosophila77,78. Alternatively, primII-derived neurastems may rely on a different subset of Wnt ligands, HSPGs or Fzd receptors to organize their support cells79,80. To date, the analysis of hair cell orientation in zebrafish mutations in two noncanonical Wnt ligands, wnt5b (pipetail) and wnt11f2 (formerly known as wnt11silverblick40), revealed no hair cell orientation defects in any primordia (Supplementary Figs. 1 and 7).

Our data suggest that tissue organization in the primordium is set up early during development and influences hair cell orientation in later stages. This mechanism is conceptually analogous to the ratchet effect proposed by Gurdon et al.81 and implies that cells have a memory of the initial conditions or signals to which they have been exposed that modulates a later response. A similar process is at work during zebrafish gastrulation, where prolonged cadherin-dependent adhesion initiates Nodal signaling and feeds back to positively increase adhesion and determines mesoderm vs. endoderm fate82. In addition, reports from cells remembering the spatial geometry or PCP that precedes cell division using the extracellular matrix or tricellular junctions support this hypothesis83–85. The memory of early polarity establishment might explain why primII-derived Wnt pathway mutant neurastems possess a phenotype, even though wnt11 (wnt11f1) is not expressed in surrounding tissues at that stage (Figs. 1 and 5).

Rather than acting as an instructive cue, Wnt pathway genes might be acting as permissive factors by controlling cell–cell adhesion. wnt11 (wnt11f1) destabilizes apical cell–cell junctions in the epithelium during pharyngeal pouch formation in zebrafish86, and expression of dominant-negative Wnt11 in Xenopus shows that Wnt11 controls cell adhesion through cadherins87. Interestingly, wnt11f2 interacts with **gpc4** and *fzd7a/b* and influences E-cadherin-dependent cell–cell adhesion, rather than PCP during zebrafish CE and eye formation19,50,88. However, even though wnt11 (wnt11f1) binds to the same receptors as wnt11f2, wnt11f2 does not affect hair cell development, suggesting that the two wnt11 orthologs likely control different adhesion molecules in the different tissues.

Another intriguing possibility is that the Wnt pathway genes might act through the Fat-Dachsous (Ft-Ds) pathway. In flies, disruptions in Ft-Ds signaling cause swirling wing hair patterns and disruptions of the global alignment of PCP proteins without affecting their asymmetric distribution, reminiscent of the phenotypes we observed in Wnt pathway mutants5,6,89–92. Furthermore, a recent report shows that the Ft-Ds pathway directs the uniform axial orientation of cells in the Drosophila abdomen93, which may be similar to the coordinated organization observed in support cells in our study. It is likely that wnt11 (wnt11f1), *gpc4* and *fzd7a/b* establish support cell organization by controlling their adhesive properties. However, the presence of a large number of adhesion molecules, and potential for functional compensation, makes functional analyses challenging.

In summary, we identified and characterized the Wnt pathway genes wnt11 (wnt11f1), gpc4 and fzd7a/b, and the PCP gene scrib as distinct inputs controlling hair cell orientation in the lateral line of zebrafish. The core PCP components **vangl2** and **scrib** control individual hair cell polarity, while the Wnt pathway genes wnt11 (wnt11f1)/**gpc4**/*fzd7a/b* affect the alignment of support cells, which are the progenitors of the later forming hair cells (Fig. 7). This interpretation is supported by our findings that PCP mutants show hair cell orientation defects in the presence of normally aligned surrounding support cells. Conversely, in Wnt pathway mutants support cells are misaligned, even in the absence of hair cells. Importantly, scRNA-Seq analysis of neuromasts shows that *gpc4*, *fzd7a* and *fzd7b* are robustly expressed in support but not hair cells, whereas **vangl2** is strongly expressed in hair cells. In addition, because the affected support cells in Wnt pathway mutants are only exposed to wnt11 (wnt11f1) ligand while the primordia are still in the head region, we propose that Wnt pathway genes act early during development before the hair cells appear.

Our study provides an alternative mechanistic model in which hair cell orientation defects are not only caused by disruptions of the PCP pathway but independently also by other pathways, such as Wnt11 (Wnt11f1)-activated Wnt signaling. The precise molecular mechanism by which Wnt signaling affects hair cell orientation is unknown but it possibly affects adhesion between support cells required to establish or maintain hair cell alignment.

**Methods**

**Fish husbandry.** This study was conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the NIH and a protocol approved by the Institutional Animal Care and Use Committees of the Stowers Institute (TP Protocol: # 2017-0176).

All experiments were performed per guidelines established by the Stowers Institute IACUC review board. The following mutant fish strains previously described were used: **myo6b**: **Actin-GFP**19,0, Tg(myo6b:Actin-GFP)19,0, Tg(myo6b:Actin-GFP)19,0,Et(krt4:EGFP)sqET4 and Et (krt4:EGFP)sqET4. The following transgenic fish lines were used: Tg(cldnb:lynGFP)ps506 99, Tg (myob58-Anti-GFP)99, Tg(cxtr5:H2A-EGFP)99,101,102, Tg(krt4:EGFP)ps506 99,101,102, Tg(cldnb:lynGFP)ps506 99, Tg (cldnb:lynGFP)ps506 99, Tg(cldnb:lynGFP)ps506 99, Tg(cldnb:lynGFP)ps506 99, Tg(cldnb:lynGFP)ps506 99, Tg(cldnb:lynGFP)ps506 99, Tg(cldnb:lynGFP)ps506 99.

**In situ hybridization.** Samples were fixed in 4% PFA overnight at 4 °C. Samples were then dehydrated in PBS/Tween 0.3% (PBSTw) and a Methanol series (0, 25, 50, 75 and 100%) for 10 min each and kept overnight at −20 °C in 100% Methanol. Then, embryos were rehydrated in PBSTw and a Methanol series and incubated with Proteinase K (10 µg/mL in PBSTw) for 2 min at RT. The fish were then postfixed in 4% PFA in PBS for 30 min. After postfixation, the larvae were incubated in Hybridization mix for 2 h at 65 °C, and then were hybridized with the Probes overnight at 65 °C. The following day the unbound probes was removed using a series of washes (50% Formamid/50% 2× SSC; 50% Formamid/5× SSC/
Support cell polarity

The following day, incubated with Blocking Solution (2% NGS, 1% BSA in PBS) for 2 h. Fish were then incubated with the secondary antibody (1:250 in Blocking Solution) for 2 h at RT. Neomycin treatment embryos were washed thoroughly using PBSTx 0.5% and imaged.

Phalloidin (Thermo Fisher; 1:40 in PBSTx 0.5%) for 2 h. After staining, the temperature for at least 2 h. PFA-fixed embryos were washed in 1× PBS and then permeabilized in 2% PBSTx for 2 h at room temperature. Subsequently, the embryos were stained with Alexa Fluor 488 Phalloidin (Thermo Fisher; 1:40 in PBSTx 0.5%) for 2 h. After staining, the embryos were washed thoroughly using PBSTx 0.5% and imaged.

Neomycin treatment. To kill hair cells, 5 dpf embryos were treated with 300μM Neomycin in 0.5 E2 media for 30 min. Neomycin was then washed away using fresh E2 media, and waited 3 h before fixation in 4% PFA in 1× PBS.

Immunohistochemistry and Phalloidin staining. For β-II-Spectrin, ZO-1 and Vangl2 antibody stainings, embryos were fixed in 2% trichloroacetic acid (TCA) in water at room temperature for at least 4 h. Embryos were then washed briefly in 0.5% Triton-X in PBS (PBSTx) and then blocked for 2 h in PBSTx + 2% normal goat serum (NGS). Primary antibodies Rabbit anti-Vangl2 (1:100, AnaSpec, AS-55659, now discontinued), Mouse anti ZO-1 (1:200, Invitrogen #339100), Mouse anti β-II-Spectrin (1:200, BD Transduction, 612562) were diluted in Blocking solution and incubated overnight at 4 °C. Primary antibody was washed using PBSTx and then detected using either Alexa-568 Anti-Rabbit and Alexa-488 Anti-Mouse Secondary antibodies for the double β-II-Spectrin/Vangl2-2 or Alexa-594 Anti-Mouse (Thermo Fisher) for the double β-II-Spectrin/ZO-1 diluted 1:500 in Blocking Solution, and incubated overnight at 4 °C. Secondary antibody was washed thoroughly the next day using PBSTx before imaging.

For Emx2 immunolabeling 5 dpf embryos were fixed in 4% PFA overnight at 4 °C. Then, embryos were permeabilized in acetone for 5 min at −20 °C and incubated with Blocking Solution (2% NGS, 1% BSA in PBS) for 2 h. Fish were then incubated with primary antibody (rabbit anti-Emx2, K0609; Trans Genic, Japan; 1:250) overnight at 4 °C. The following day, fish were washed in 1× PBS and incubated with the secondary antibody (1:250 in Blocking Solution) for 2 h at RT. The fish were then washed thoroughly in PBS and imaged.

For Phalloidin staining, embryos were fixed in 4% PFA in 1× PBS at room temperature for at least 2 h. PFA-fixed embryos were briefly washed in 0.5% Triton-X in 1× PBS and then permeabilized in 2% PBSTx for 2 h at room temperature. Subsequently, the embryos were stained with Alexa Fluor 488 Phalloidin (Thermo Fisher; 1:40 in PBSTx 0.5%) for 2 h. After staining, the embryos were washed thoroughly using PBSTx 0.5% and imaged.

Time-lapse imaging and in vivo imaging. Embryos were anesthetized with Tricaine (MS-222) and mounted in 0.8% low-melting point agarose in glass bottom dishes (MatTek, USA). Embryos were imaged with a Zeiss 780 confocal microscope using a 40×/1.1 W Corr M27 objective in a climate-controlled chamber set to 28 °C.

Hair cell orientation analysis. To calculate the hair cell orientation from the Phalloidin-stained neuromasts, the Line tool from Fiji was used. A straight line was drawn from the pole opposite to the kinocilium towards the pole were the kinocilium was found. To create the Rose diagrams to represent hair cell orientation, angles measured and then plotted using the rose.diag() function in R ggplot. Statistical analysis was performed for each condition against wild type using Fisher’s exact test. To determine A-P or D-V binomial distribution, the data are binned to four groups, left, right, bottom, and up. The binomial test is used to test whether ‘right ≠ left’ counts are different from 50% of total counts.

Measure of hair cell alignment and concentricity. We must choose a structure against which to compare the cell’s angles. Specifically, we are looking at relative differences in the angles and an assumed underlying arrangement, rather than comparing them to some absolute feature, such as the horizontal. The first comparison we consider is the angular difference between nearest neighbors. Note although it is possible to provide a consistent polarization of the cell and, thus, measure the angle between two nearest major axes on a scale of (−180, 180)° we are not currently interested in the polarization, thus, we always take the smallest angle between the two major axes. Hence, the angles will be on a scale of (−90, 90)°. Further, the sign convention we will be using is positive if the angle measured from the current cell is anticlockwise, and negative otherwise. The second comparison we consider is cell alignment when compared to a fitted ellipse. Initially, we take the cell centers of the Lines calculated in the “Hair cell orientation analysis” section, and use a least squares fitting algorithm based on principal component analysis to create an ellipse of best fit. Each cell is then projected to the closest point on the fitted ellipse’s boundary and their major axis angle is compared to that of the ellipse’s tangent at the nearest point. The smallest angle is extracted using the above sign rule. The “Nearest neighbor measurement” provides us with a measurement of how aligned each cell is with its neighbors, while the “Nearest tangent measurement” will provide us with a measurement of how aligned each cell is with the fitted ellipse. Specifically, this comparison will suggest whether there is a circular structure underlying the cell alignment. Having derived the data from the experimental results we plot the information as a histogram. Specifically, the range (−90, 90)° is divided up into 12 bins and we tally how often each angle falls within one of these bins. Dividing by the total number of results then normalizes the histograms to represent a probability. Each angle distribution was first tested against the null hypothesis that there was no preferred direction. Thus, we compare the probability histogram against the uniform distribution and see if there was a significant difference, namely p < 0.01. If there was a significant difference we then compared the solutions to the Von Mises distribution, which is the generalized
Normal distribution that is periodic on the angular domain. The fitting of the Von Mises distribution tells us if there is a preferred direction and how variable the alignment is. The code for these analyses is publicly available at https://github.com/ThomasEWoolley/Cell_alignment.

**Vang2 asymmetry quantification.** Vang2 asymmetry quantifications from immunolabeled embryos were performed from raw images. Upon visual inspection, Vang2 being “asymmetric” vs. “not being asymmetric” was assigned and quantified using the Cell Counter function of FIJI. Fisher’s exact test was performed to determine statistical differences between samples. From the hair cells that showed Vang2 asymmetry, a second analysis was performed and classified into three categories: we quantified whether the enrichment was (1) in the pole where the kinocilium is, (2) on the opposite pole, or (3) enriched but out of the axis determined by the kinocilium. Fisher’s exact test was performed to determine statistical differences between samples. The double immunolabeled images were processed for contrast and sharpness only for visual display in the Figure afterwards using Photoshop CS6.

**Generation of the Tg(myo6b:GFP-XVangl2) construct.** GFP-XVangl2 was amplified from pDest2-GFP-XVangl2 plasmid (A kind gift from J. Wallingford) and Topo TA cloned. The Topo vector was then digested using KpnI and SacI, and ligated into the KpnI-SacI sites of the To2 middle entry vector105 using Quick ligase (NEB). The middle entry GFP-XVangl2 vector was recombined via a Gateway reaction with the 5′ myob vector106. The final myob-GFP-XVangl2 plasmid was co-injected into AB or MZwnt11 (wt11f1) mutant embryos together with To2 Transposase mRNA at a final concentration of 50 ng/μl.

**GFP-XVangl2 profile analysis.** Images were acquired in a Zeiss 780 microscope using a 40x/1.1 WCorr M27 objective. Raw images of Phalloidin-stained animals were used for the analysis of GFP intensity. GFP-XVangl2 polarity was determined in a manner analogous to111. These utilized custom plugins as described above. So far, cortical line profiles were generated with a 4 pixel thickness utilizing the “polypine kymograph” plugin. Then maximum angular position of intensity was determined using the “trajectory statistics jr v2” plugin. Profiles were then aligned so that the maximum intensity occurred at angle 0 with “set multi plot offsets jr v1”. Next, angles were wrapped to values between −179 and +180 degrees with “wrap angle profile jr v1”. Finally, profiles were averaged with “average trajectories jr v1” (available at http://research.stowers.org/imagejplugins/) with error bars indicating the standard error in the mean. For some presentations, averaged profiles were normalized to a maximum intensity of 1 for comparison purposes. Peak widths were determined by fitting profiles to a Gaussian peak function via nonlinear least squares fitting (plugin is “fit multi gaussian jr v1”) with error bars determined by fitting 100 Monte Carlo simulated random data sets112. Polarity ratios were determined by averaging three values surrounding the 0°, 90°, and 180° data points and calculating their ratios with errors propagated according to ref. 112.

**Hair cell progenitor angle of division analysis.** Imaging was performed in a Zeiss 780 using a 40x/1.1 WCorr M27 objective, under conditions described above (Time-lapse imaging section). To obtain a quantitative measurement of how hair cell progenitors divide on the neuromast, we acquired time-lapse images of migrating prmlf in wild type and MZwnt11 (wt11f1) mutant embryos in a t/pcxcr4-H2A-EGFP, t/myobb-Actin-GFP double background, scanning every 5 min. We calculated the division angle of the dividing progenitor with respect to the radius from the center of division, to the center of the neuromast.

We used Imaris v64 9.2.1 to manually mark the location of all cell divisions and the center point of the depositing neuromast for all time-points. To find the center of the neuromast we create a Surface object using the H2A-EGFP channel with very high contrast and then used the “slicer” tool to identify the surface points in the 3D image and stitched afterwards using the Morphol plugin113. Once a mask of the cells within the neuromast was obtained, we found the orientation of each cell using FIJI’s “Analyze Particles” function. This function fits each cell to an ellipse in order to provide spatial data, including the center point, major and minor axis length, and the major and minor axis angle in cartesian coordinates. We exported the data from FIJI to Python where we processed and plotted the data. We used Matplotlib to create a histogram of the distribution of cell orientation angles in the range of 0°–90°. To visualize and verify the data, we also plotted the major axis vector on top of the cell mask image for each sample. The code for this analysis is publicly available at https://github.com/richard-alexander/neuromast_cell_orientation.

**vang1 CRISPR.** We designed two guide RNAs using CRISPRscian (www.crisprscian.org)114 targeting exon 3 of vang1 (ENSEMBL:G000000004305). The guide-RNA plus PAM sequences are 5′-GGTTCAGTGACGCGCCGTTGGG-3′ and 5′-GGCTTTGGCAAAGAACACGGAAGG-3′ in vitro transcription using a Mega-Shortscript T7 kit (Invitrogen) was carried out the manufacturer’s recommended conditions. RNA was purified using a RNA Clean and Concentrator kit (Zymo Research). One-cell stage embryos were injected with an injection mixture containing 1 μg of Cas9-NLS Protein (PNABio) with 170 ng of guide-RNA RNA. We assessed cutting efficiency, the region around the cutting sites was amplified from genomic DNA by PCR using 5′GAAGATGCAGCTATTTGCTTAAATCG-3′ and 5′-TAGTTAGAGCTTGAGTGAGAATAATG-3′ and amplified by PCR. From the resulting DNA template, the in vitro reaction was carried out with the T7 MEGASHortscript kit (Invitrogen) under manufacturer conditions, and the RNA was purified and concentrated using the RNA Clean & Concentrator-25 kit (Zymo, #MR1017). A mixture of 30 ng of each gRNA was mixed with 1 μg of recombinant Cas9-NLS protein (PNABio, CP04-100) and injected into one-cell stage embryos. To assess the success of the injection, 5 dpf CRISPant embryos were stained with DASPEI (2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide, [Invitrogen, USA]) diluted in embryo media and screened for the lack of hair cells compared with the uninjected siblings. To assess cutting efficiency, the region around the cutting sites was amplified from genomic DNA by PCR using ATGCATAGGCAGTAGAT and ATGTTAGCTGCGACGCTGAC as primers and run on an agarose gel.

**Single-cell RNA-Seq.** The scRNA-Seq has been described in detail in66. The BAM files and count matrices produced by cell ranger can be accessed in the Gene Expression Omnibus (GEO) database (accession number GSE123241). Together with this paper we published a publicly available, searchable database that allows the user to determine in which neuromast cell type candidate genes are expressed: https://piorowskilab.shinyapps.io/neuromast_homeostasis_scsrnaseq_2018/.

**Supporting information.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The authors declare that all data supporting the findings of this study are available within the article and supplemental information files or upon reasonable request. The source data underlying Figs. 1, 2, 3 and Supplementary Figs. 2, 4 and 7 are provided as a Source Data file. Uncropped blots for Supplementary Figs. 2 and 5 are also provided in the Source data file. This data has also been deposited in the Stowers Institute Original Data Repository and available online at http://www.stowers.org/research/publications/lhpdb-1424.

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**References.**

1. Goodrich, L. V. & Strutt, D. Principles of planar polarity in animal development. Development 138, 1877–1892 (2011).
2. Butler, M. T. & Wallingford, J. B. Planar cell polarity in development and disease. Nat. Rev. Mol. Cell Biol. 18, 375–388 (2017).
3. Devenport, D. The cell biology of planar cell polarity. J. Cell Biol. 207, 171–179 (2014).
4. Strutt, H. & Strutt, D. Long-range coordination of planar polarity in Drosophila. Bioessays 27, 1218–1227 (2005).
5. Lawrence, P. A. & Shelton, P. M. The determination of polarity in the developing insect retina. J. Embryol. Exp. Morphol. 33, 471–486 (1975).
6. Gubb, D. & García-Bellido, A. A genetic analysis of the determination of cuticular polarity during development in Drosophila melanogaster. J. Embryol. Exp. Morphol. 68, 37–57 (1982).

**Reporting summary.**
7. Wong, L. L. & Adler, P. N. Tissue polarity genes of Drosophila regulate the subcellular location for prehair initiation in pupal wing cells. J. Cell Biol. 123, 221–231 (1993).
8. Lawrence, P. A. Development and determination of hairs and bristles in the milkweed bug, Oncopeltus fasciatus (Lygaeidae, Hemiptera). J. Cell Sci. 1, 475–498 (1966).
9. Tada, M. & Smith, J. C. Wnt11 is a target of Xenopus Brachyury: regulation of cell specification movements via Dishevelled, but not through the canonical Wnt pathway. Development 127, 2227–2238 (2000).
10. Wallingford, J. B. et al. Dishevelled controls cell polarity during Xenopus gastrulation. Nature 405, 81–85 (2000).
11. Heisenberg, C. P. et al. Silberblick/Wnt11 mediates convergent extension movements of zebrafish gastrulation. Nature 405, 76–81 (2000).
12. Gao, B. Wnt regulation of planar cell polarity (PCP). Curr. Top. Dev. Biol. 101, 263–295 (2012).
13. Yang, Y. & Mlodzik, M. Wnt-Desfilized/planar cell polarity signaling: cellular orientation by facing the wind (Wnt). Annu. Rev. Cell Dev. Biol. 31, 623–646 (2015).
14. Humphries, A. C. & Mlodzik, M. From instruction to output: Wnt/PCP signaling in development and cancer. Curr. Opin. Cell Biol. 25, 110–116 (2018).
15. Wu, J., Roman, A. C., Carvajal-Gonzalez, J. M. & Mlodzik, M. Wg and Wnt4 promote long-range directional input to planar cell polarity orientation in Xenopus laevis. Development 138, 2129–2138 (2011).
16. Witzel, S., Zimyanin, V., Carreira-Barbosa, F., Tada, M. & Heisenberg, C. P. Wnt11 controls cell contact persistence by local accumulation of Frizzled 7 at plasma membranes. J. Cell Sci. 175, 791–802 (2007).
17. Ulrich, F. et al. Wnt11 functions in gastrulation by controlling cell cohesion through RhoA and C–adherin. Dev. Cell 6, 555–564 (2005).
18. Gordon, L. R., Gribble, K. D., Syrett, C. M. & Granato, M. Initiation of synapse formation by Wnt-induced MusK endocytosis. Development 139, 1023–1033 (2012).
19. Deans, M. R. A balance of form and function: planar polarity and development of the vestibular maculae. Semin. Cell Dev. Biol. 24, 490–498 (2013).
20. May-Smera, H. & Kelley, M. W. Planar cell polarity in the inner ear. Curr. Top. Dev. Biol. 101, 111–140 (2012).
21. Dahdouh, A. et al. Wnt signaling mediates reorientation of outer hair cell stereociliary bundles in the mammalian cochlea. Development 130, 2375–2384 (2003).
22. Qian, D. et al. Wnt5a functions in planar cell polarity regulation in mice. Dev. Biol. 306, 121–133 (2007).
23. Ghysen, A. & Dambly-Chaudiere, C. The lateral line microcosmos. Curr. Opin. Cell Biol. 21, 2118–2130 (2009).
24. Pujol-Martí, J. & Lopez-Schier, H. Developmental and architectural principles of the lateral-line neural map. Front. Neural Circuits 7, 47 (2013).
25. Venero Galanternik, M., Navajas Acedo, J., Semenza, A. et al. The zebrafish gpa1 and gpa2 genes regulate the regulation of convergent-extension movements during zebrafish gastrulation. Dev. Dyn. 242, 1045–1055 (2013).
26. Gao, B. Wnt regulation of planar cell polarity (PCP). Curr. Top. Dev. Biol. 101, 263–295 (2012).
27. Wu, J., Roman, A. C., Carvajal-Gonzalez, J. M. & Mlodzik, M. Wg and Wnt4 promote long-range directional input to planar cell polarity orientation in Xenopus laevis. Development 138, 2129–2138 (2011).
28. Roche, T. et al. Light-activated Frizzled7 reveals a permissive role of non-canonical Wnt signaling in mesendoderm cell migration. eLife 6, e40293 (2017).
29. Ferraro, M. E. et al. Wnt7b signalling through Frizzled-7 receptor promotes dendrite development by coactivating CaMKII and JNK. J. Cell Sci. 131, jcs216101 (2018).
30. Nemeth, D. S., Usmani, M., Pawlicki, S. & Solnica-Krezel, L. Wnt/PCP signaling controls intracellular position of MTOCs during gastrulation convergence and extension movements. Development 138, 543–552 (2011).
31. Montcouquioil, M. et al. Identification of Vang2 and Scrb1 as planar polarity genes in mammals. Nature 423, 173–177 (2003).
32. Montcouquioil, M. et al. Asymmetric localization of Vang2 and Fzd3 indicate novel mechanisms for planar polarity in mammalian cells. J. Neurosci. 26, 5265–5275 (2006).
33. Stoller, M. L., Roman, O. Jr. & Deans, M. R. Dominating non-autonomy in Vang1/Vang2 double mutants demonstrates intercellular PCP signaling in the vertebrate inner ear. Dev. Biol. 437, 17–26 (2018).
34. Yin, H., Copley, C. O., Goodrich, L. V. & Deans, M. R. Comparison of phenotypes between different vang2 mutants demonstrates dominant effects of the Looptail mutation during hair cell development. PLoS ONE 7, e31988 (2012).
35. Hammerschmidt, M. et al. Mutations affecting morphogenesis during gastrulation and tail formation in the zebrafish, Danio rerio. Development 123, 153–151 (1996).
36. Jessen, J. R. et al. Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. Nat. Cell Biol. 4, 610–615 (2002).
37. Lu, M. E. & Piotrowski, T. Sensory hair cell regeneration in the zebrafish lateral line. Dev. Dyn. 243, 1187–1204 (2015).
38. Romero-Carvajal, A. et al. Sensory hair cell regeneration requires localized interactions between the Notch and Wnt Pathways. Dev. Cell 34, 267–282 (2015).
39. Harris, J. A. et al. Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (Danio rerio). J. Assoc. Res. Otolaryngol. 4, 219–234 (2003).
40. Jessen, J. R. et al. Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. Nat. Cell Biol. 2, 610–615 (2002).
41. Romero-Carvajal, A. et al. Sensory hair cell regeneration in the zebrafish lateral line. Dev. Dyn. 243, 1187–1204 (2015).
42. Hammerschmidt, M. et al. Mutations affecting morphogenesis during gastrulation and tail formation in the zebrafish, Danio rerio. Development 123, 153–151 (1996).
43. Jessen, J. R. et al. Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. Nat. Cell Biol. 4, 610–615 (2002).
44. Lu, M. E. & Piotrowski, T. Sensory hair cell regeneration in the zebrafish lateral line. Dev. Dyn. 243, 1187–1204 (2015).
45. Romero-Carvajal, A. et al. Sensory hair cell regeneration requires localized interactions between the Notch and Wnt Pathways. Dev. Cell 34, 267–282 (2015).
46. Harris, J. A. et al. Neomycin-induced hair cell death and rapid regeneration in the lateral line of zebrafish (Danio rerio). J. Assoc. Res. Otolaryngol. 4, 219–234 (2003).
47. Jessen, J. R. et al. Zebrafish trilobite identifies new roles for Strabismus in gastrulation and neuronal movements. Nat. Cell Biol. 2, 610–615 (2002).
64. Jiang, L., Romero-Carvajal, A., Haug, J. S., Seidel, C. W. & Piotrowski, T. Gene-expression analysis of hair cell regeneration in the zebrafish lateral line. Dev. Natl Acad. Sci. USA 111, E1383–E1392 (2014).

65. Jing, L., Lefebvre, J. L., Gordon, L. R. & Granato, M. Wnt signals organize synaptic prepattern and axon guidance through the zebrafish unplugged/Msuk receptor. Neuron 61, 721–733 (2009).

66. Lush, M. E. et al. scRNA-Seq reveals distinct stem cell populations that drive hair cell regeneration. Dev. Cell 48, 129–139 (2021).

67. Jones, C. et al. Ankrd6 is a mammalian functional homolog of Drosophila planar cell polarity gene diego and regulates coordinated cellular orientation in the mouse inner ear. Dev. Biol. 395, 62–72 (2014).

68. Deans, M. R. et al. Asymmetric distribution of prickle-like 2 reveals an early underlying polarization of vestibular sensory epithilia in the inner ear. J. Neurosci. 27, 3147–3157 (2007).

69. Wang, J. et al. Regulation of polarized extension and planar cell polarity in the cochlea by the vertebrate PCP pathway. Nat. Genet. 37, 980–985 (2005).

70. Duncan, J. S. et al. Celsr1 coordinates the planar polarity of vestibular hair cells during inner ear development. Dev. Biol. 423, 126–137 (2017).

71. Sienknecht, U., J., Anderson, B. K., Parodi, R. M., Fantetti, K. N. & Fekete, D. M. Non-cell-autonomous planar cell polarity propagation in the auditory sensory epithelium of vertebrates. Dev. Biol. 352, 27–39 (2011).

72. Jiang, T., Kindt, K. & Wu, D. K. Transcription factor Ermx2 controls stereociliary bundle orientation of sensory hair cells. eLife 6, e23661 (2017).

73. Sapede, D., Gompel, N., Dambly-Chaudiere, C. & Ghysen, A. Cell migration determines the axis of planar polarity in ciliated epithelia. Dev. Cell 6, 231–242 (2004).

74. Aw, W. Y., Beck, B. W., Joyce, B. & Devenport, D. Transient tissue-scale deformation coordinates alignment of planar cell polarity junctions in the mammalian skin. Curr. Biol. 26, 2090–2100 (2016).

75. Chien, Y. H., Srinivasan, S., Keller, R. & Kintner, C. Mechanical strain determines cilia, motility, and planar orientation in the left-right organizer. Dev. Cell 45, 316–330 e314 (2018).

76. Chien, Y. H., Keller, R., Kintner, C. & Shoock, D. R. Mechanical strain determines the axis of planar polarity in ciliated epithelia. Curr. Biol. 25, 2774–2784 (2015).

77. Olguin, P., Clavio, A. & Molinik, M. Intertissue mechanical stress affects Frizzled-mediated planar cell polarity in the Drosophila notch epidermis. Curr. Biol. 21, 236–242 (2011).

78. Aigouy, B. et al. Cell flow reorients the axis of planar polarity in the wing epithelium of Drosophila. Cell 142, 773–786 (2010).

79. Escobedo, N. et al. Syndecan 4 interacts genetically with Vangl2 to regulate neural tube closure and planar cell polarity. Development 140, 3008–3017 (2013).

80. Wang, Y., Guo, N. & Nathans, J. The role of frizzled3 and frizzled6 in neural tube closure and in the planar polarity of inner-ear sensory hair cells. J. Neurosci. 26, 2147–2156 (2006).

81. Gurdon, J. B., Mitchell, A. & Mahony, D. Dynamic and continuous assessment by cell tracking of their position in a morphogen gradient. Nature 376, 520–521 (1995).

82. Barone, V. et al. An effective feedback loop between cell-cell contact duration and morphogen signaling determines cell fate. Dev. Cell 43, 198–211 e112 (2017).

83. Devenport, D., Oristan, D., Heller, E. & Fuchs, E. Mitotic internalization of planar cell polarity proteins preserves tissue polarity. Nat. Cell Biol. 13, 895–902 (2011).

84. Thery, M. et al. The extracellular matrix guides the orientation of the cell division axis. Nat. Cell Biol. 7, 947–953 (2005).

85. Bosveld, F. et al. Epithelial tricellular junctions act as interphase cell shape sensors to orient mitosis. Nature 530, 495–498 (2016).

86. Choe, C. P. et al. Wnt-dependent epithelial transitions drive pharyngeal pouch formation. Dev. Cell 24, 296–309 (2013).

87. Dzambara, B. I., Jakab, K. R., Marsden, M., Schwartz, M. A. & DeSimone, D. W. Cadherin adhesion, tissue tension, and noncanonical Wnt signaling regulate cell polarity during collective cell migration. Cell 160, 414–425 (2015).

88. Kอกkaidó, M. et al. Proliferation-independent regulation of organ size by Fgf/Notch signaling. eLife 6, e21049 (2017).

89. Parnov, S., Kondrichin, I., Korzh, V. & Emelyanov, A. Toll2 transposon-mediated enhancer trap to identify developmentally regulated zebrafish genes in vivo. Dev. Dyn. 231, 449–459 (2004).

90. Lush, M. E. & Piotrowski, T. ErbB expressing Schwann cells control lateral progenitor cells via non-cell-autonomous regulation of Wnt/beta-catenin. eLife 3, e01832 (2014).

91. Ma, E. Y., Rubel, E. W. & Raible, D. W. Notch signaling regulates the extent of hair cell regeneration in the zebrafish lateral line. J. Neurosci. 28, 2261–2273 (2008).

92. Kopinke, D., Sasanov, J., Zvi, J., Stephens, W. Z. & Piotrowski, T. Retinoic acid is required for endodermal pouch morphogenesis and not for pharyngeal endoderm specification. Dev. Dyn. 235, 2095–2709 (2006).

93. Zupan, K. J. & Piotrowski, T. Heparan sulfate proteoglycans regulate Fgf signaling and cell polarity during collective cell migration. Cell Rep. 10, 414–428 (2015).

94. Nikaido, M., Law, E. W. & Kelsh, R. N. A systematic survey of expression and function of zebrafish frizzled genes. PLoS One 8, e54833 (2013).

95. Schindelin, I., et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).

96. Butler, M. T. & Wallingford, J. B. Spatial and temporal analysis of PCP protein dynamics during neural tube closure. eLife 7, e36456 (2018).

97. Kwan, K. M. et al. The Tol2kit: a multisite gateway-based construction kit for CRISPR/Cas9-mediated enhancer trap to identify developmentally regulated zebrafish genes in vivo. Development 139, 1011–1016 (2012).

98. Bevington, P. R. & Robinson, D. K. Data Reduction and Error Analysis for the Physical Sciences 3rd edn (McGraw-Hill, 2003).

99. Klingenberg, C. P. MorphoJ: an integrated software package for geometric morphometrics. Mol. Ecol. Resour. 11, 353–357 (2011).

100. Moran, M. et al. CRISPRscan: designing highly efficient sgRNAs for CRISPR-Cas9 targeting in vivo. Nat. Methods 12, 982–988 (2015).

101. Montague, T. G., Cruz, J. M., Gagnon, J. A., Church, G. M. & Valen, E. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. Nucleic Acids Res. 42, W401–W407 (2014).

102. Vardhaney, G. K. et al. High-throughput gene targeting and phenotyping in zebrafish using CRISPR/Cas9. Genome Res. 25, 1030–1042 (2015).
