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Report

The PCP Pathway Instructs the Planar Orientation of Ciliated Cells in the *Xenopus* Larval Skin

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Summary

Planar cell polarity (PCP) is a property of epithelial tissues where cellular structures coordinately orient along a two-dimensional plane lying orthogonal to the axis of apical-basal polarity [1]. PCP is particularly striking in tissues where multiciliate cells generate a directed fluid flow, as seen, for example, in the ciliated epithelia lining the respiratory airways or the ventricles of the brain. To produce directed flow, ciliated cells orient along a common planar axis in a direction set by tissue patterning, but how this is achieved in any ciliated epithelium is unknown [2]. Here, we show that the planar orientation of *Xenopus* multiciliate cells is disrupted when components in the PCP-signaling pathway are altered non-cell-autonomously. We also show that wild-type ciliated cells located at a mutant clone border reorient toward cells with low Vangl2 or high Frizzled activity and away from those with high Vangl2 activity. These results indicate that the PCP pathway provides directional non-cell-autonomous cues to orient ciliated cells as they differentiate, thus playing a critical role in establishing directed ciliary flow.

Results and Discussion

Planar cell polarity (PCP) has been extensively studied in *Drosophila*, where it is evident in the ordered projection of hairs on the wing and abdomen or in the orientation of ommatidia in the eye [3]. The genes required to orient these structures include those encoding the core components of the PCP-signaling pathway—namely three intracellular proteins, Prickle, Dishevelled, and Diego, and three transmembrane proteins, Flamingo, Frizzled, and Van Gogh (also known as Strabismus) (reviewed in [4–6]). In genetic mosaics, two of these genes, *Frizzled* and *Van Gogh*, produce profound non-cell-autonomous phenotypes in which the orientation of wild-type cells adjacent to a mutant clone is redirected inward or outward [6–8]. In addition, Frizzled and Van Gogh dynamically accumulate during PCP signaling at opposite sides of a polarized cell (reviewed in [4]). These and other observations suggest that Frizzled and Van Gogh, in combination with Flamingo, act as directional cues to align cells along a planar axis based on local cell-to-cell comparisons [9–11]. PCP signaling is also conserved in vertebrates. Homologs of several PCP components are known to localize asymmetrically within polarized cells, such as cochlear hair cells, and to disrupt PCP in several tissues when mutant (reviewed in [5, 12]).

The role of the PCP pathway in orienting ciliary flow has been studied in the *Xenopus* larval skin by using morpholinos to knock down the cytoplasmic PCP component, Dishevelled, or two downstream effectors of PCP called inturned and fuzzy [13, 14]. When all three *Xenopus* Dishevelled homologs are targeted by morpholinos (Dvl1-3), basal bodies (BBs) fail to dock at the apical surface and cilia are lost, a phenotype also observed in morphants of *inturned* and *fuzzy*. Though this phenotype is not a defect in PCP per se, it does suggest that PCP components are required in a targeting mechanism that localizes and docks BBs at the apical membrane where cilia outgrowth occurs. However, Dishevelled function can also be disrupted in embryos by expressing a well-characterized, dominant-negative mutant of Dvl2 called Xdd1 [14]. In these embryos, cilia now form and beat but fail to polarize along a planar axis, suggesting that Dishevelled also functions downstream of BB docking in a mechanism that establishes their rotational orientation. Because the Dishevelled proteins have functions outside of the PCP pathway [15, 16], it remains unclear whether cell-cell interactions involving the PCP pathway are required to align ciliated cells along a planar axis.

To address the role of cell-cell interactions in orienting ciliated cells, we exploited how these cells arise and are patterned during *Xenopus* skin development [17]. Classic grafting experiments in other amphibian species have shown that the direction of ciliary flow along the anterior to posterior axis (A-P) is set by a patterning event that occurs soon after gastrulation and prior to ciliated cell differentiation [18, 19]. At this stage, the developing skin in *Xenopus* embryos is not one epithelial layer as found in other amphibians but is two layered, and the ciliated cells arise as precursors in the inner layer before intercalating into the outer epithelial layer. Thus, if the global axis of planar polarity is also fixed this early in the *Xenopus* skin, then ciliated cell precursors presumably acquire an orientation when they intercalate, based on cues established earlier in the outer epithelium. To confirm when the A-P polar axis is set in the skin of *Xenopus laevis*, we rotated a small patch of developing skin before and after gastrulation, allowed the embryos to develop, and scored the subsequent orientation of ciliated cells in the graft relative to the host (Figure 1A). Cilia orientation was examined in these grafts functionally by flow measurements (Movies S1 and S2 available online) and by measuring the rotational orientation of basal bodies with a confocal assay [14]. The confocal assay measures basal body orientation by using two fusion proteins to label basal bodies with RFP and the rootlets with GFP (see Experimental Procedures and Figure 2). The results show that the planar orientation of the skin is set in *Xenopus* soon after gastrulation (Figure 1) and prior to ciliated cell differentiation, implying that the ciliated cells only acquire their planar orientation when they later join the epithelium during intercalation.

To determine whether the PCP pathway is required in outer cells to orient intercalating ciliated cells, we used a

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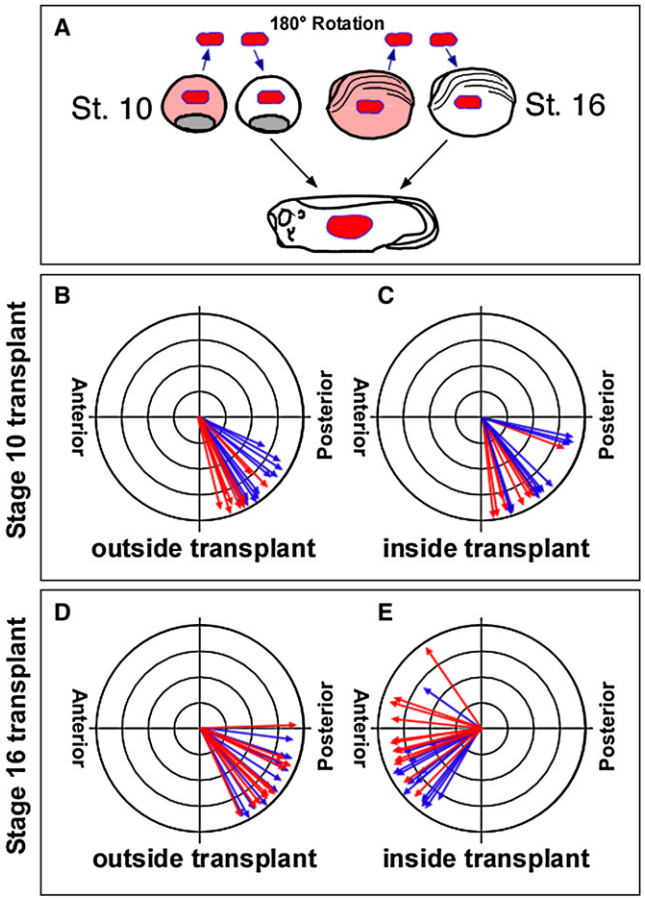

Figure 1. Timing of Planar Axis Determination

(A) Diagram of a grafting experiment in which both layers of the developing skin were isolated at the indicated stage, rotated 180°, and transplanted homotopically onto host embryos. At stage 28, the orientation of ciliated cells both inside and outside the graft was determined by confocal microscopy (see Experimental Procedures and Figure 2).

(B–E) Ciliated cell orientation following a stage 10 (B and C) or stage 16 (D and E) skin transplant and scored outside (B and D) and inside of the transplant (C and E). Each arrow represents the mean orientation of BBs within a cell, and arrow length represents the complement of circular variance around that mean. Colors represent data from separate embryos. Ciliated cells normally orient posteriorly with a ventral bias.

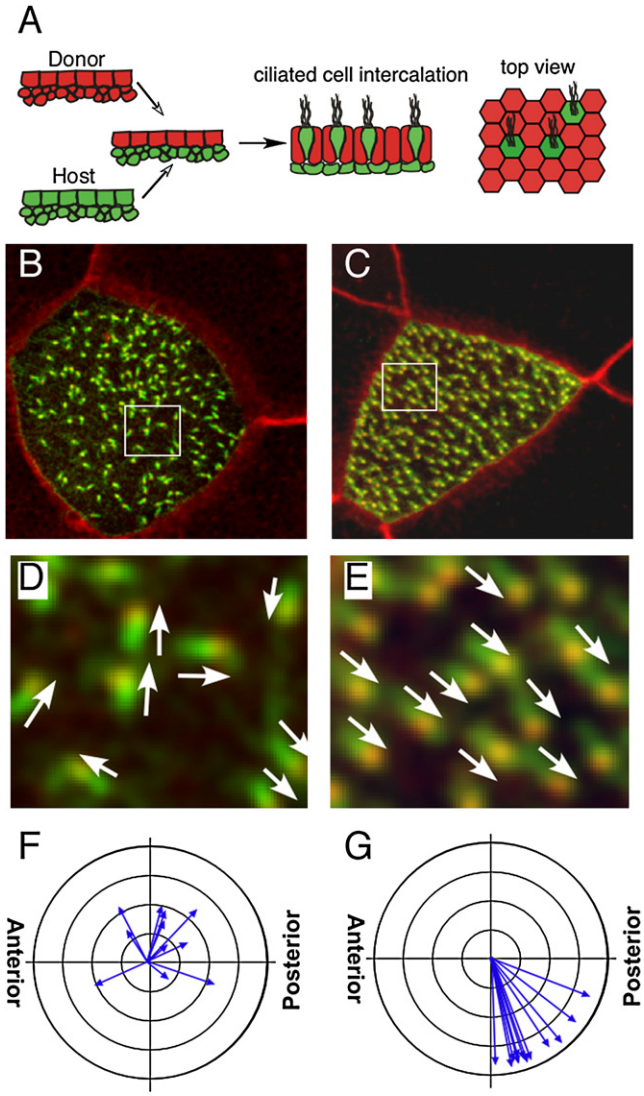

Figure 2. Xdd1 Functions Cell Autonomously to Disrupt Cilia Orientation

(A) Diagram of the assay in which outer cells are grafted from a donor onto a host embryo prior to gastrulation.

(B and C) Confocal image of an Xdd1-expressing ciliated cell surrounded by a transplant of wild-type outer cells (B) or of wild-type ciliated cells surrounded by a transplant of Xdd1-expressing outer cells (C). Ciliated cells express a Centrin-RFP (red) fusion protein that labels BBs and a CLAMP-GFP (green) fusion protein that labels the rootlet.

(D and E) The areas indicated in (B) and (C) are magnified 5× in (D) and (E), respectively. Arrows indicate the direction of cilium orientation based on centrin and CLAMP staining.

(F and G) Circular graphs depicting mean cilia orientation of cells from a transplant of wild-type outer cells onto *Xdd1*-injected ciliated cells (D) or Xdd1-injected outer cells transplanted onto wild-type ciliated cells (E). Each arrow represents the mean direction of BBs within a cell, and the length represents one minus the circular variance around the mean. Therefore, cells with short arrows vary more in BB orientation than those with long arrows. In wild-type embryos, ciliated cells beat in a posterior direction with a ventral bias.

transplantation assay to selectively disrupt the PCP pathway in ciliated cells or in the epithelia into which they intercalate (Figure 2A). This assay was first used to determine whether the Dishevelled mutant Xdd1 disrupts the rotational axis of BBs by acting solely in ciliated cells or whether it also disrupts the ability of outer cells to orient ciliated cells non-cell-autonomously [14]. When Xdd1-expressing ciliated cells intercalate into wild-type outer cells, the polar orientation of the cilia is severely disrupted, as predicted for a cell-autonomous phenotype (Figures 2B and 2D). This disorientation was evident at two levels: BB orientation was severely disorganized within cells (Figures 2B and 2D and short arrows in 2F), and the mean cilia orientation of ciliated cells failed to converge along the A-P axis (mean direction of arrows in Figure 2F). By contrast, when wild-type ciliated cells intercalated into outer epithelium expressing Xdd1, their BBs oriented normally within cells (Figures 2C and 2E and long arrows in Figure 2G), and ciliated cells were polarized in a posterior direction with a ventral bias as normal (mean direction of arrows in Figure 2G compared to Figures 1B and 1D). Thus, Xdd1 disrupts BB orientation cell autonomously but cannot disrupt ciliated cell polar orientation in a noncell-autonomous fashion.

We next asked whether the transmembrane components of the PCP pathway are required to orient ciliated cells, initially by targeting a *Xenopus* homolog of Van Gogh called Vangl2, by using a morpholino designed to block the translation of *Vangl2* RNA (Vangl2^{MO}). The ability of the Vangl2^{MO} to disrupt Vangl2 function was first tested in the mesoderm, where PCP signaling in general and Vangl2 in particular is required for the polarized cell movements that underlie axial elongation [20]. As predicted, injecting Vangl2^{MO}, but not a control MO, into the marginal zone of two-cell embryos produced strong defects in axial elongation (Figure S1). We then asked whether disrupting Vangl2 function in the skin by injecting the Vangl2^{MO} into the animal pole of two-cell embryos causes defects in ciliogenesis, as reported previously for a knockdown of Dvl1-3, Inturned, or Fuzzy [13, 14]. Indeed, in Vangl2^{MO} morphants, BBs failed to dock at the apical surface, and cilia were dramatically reduced in number. This phenotype was substantially rescued by coinjecting a synthetic *Vangl2* mRNA lacking sequences targeted by the morpholino (Figure S2). Although fewer in number, the extant cilia in Vangl2 morphants have a similar beat frequency to that of wild-type cilia but are disorganized in orientation (Figure S3 and Movies S3 and S4). Thus, these results suggest that the PCP pathway and, specifically, Vangl2 is required for BB apical localization and ciliogenesis but is unlikely to be required for cilia motility [13, 14].

Because the deleterious effects of Vangl2 on ciliogenesis are likely to be cell autonomous, as shown above for Xdd1, we next asked whether a loss of Vangl2 function in outer cells resulted in non-cell-autonomous effects on the polarity of wild-type ciliated cells (Figure 3A). As a control, ciliated cell orientation was found to be normal when a wild-type outer layer (mRFP) was transplanted onto a wild-type embryo (Figure 3, compare [B] and [C]). By contrast, when outer layer cells were transplanted from Vangl2^{MO}-injected donors onto wild-type hosts, the orientation of the intercalated wild-type ciliated cells within the clone was severely disorganized compared with cells outside the clone (Figure 3, compare [E] and [D]). Significantly, the non-cell-autonomous disruption of cilia orientation obtained in this experiment was distinct from that obtained cell autonomously with Xdd1 above. Specifically, the Vangl2 mutant outer cells did not disrupt the orientation of BBs within ciliated cells or the coordinated beating of cilia (longer arrows in Figure 3E; compare Movie S2 to S3) but, rather, the orientation of ciliated cells along the A-P axis. Indeed, ciliated cell orientation varied significantly more on average around a mean direction within a Vangl2^{MO}-injected clone compared to a control ($p = 0.000471$). These results suggest that ciliated cells acquire their orientation via cell-cell interactions with the outer layer, as they intercalate and suggest that the orientation cue requires Vangl2.

In cases in which PCP signaling has been shown to act, overexpression of components of the PCP pathway in gain-of-function experiments often causes similar polarity defects as those observed in loss-of-function experiments. Thus, to further assess the role of the PCP pathway in outer cells, we transplanted outer cells overexpressing *Vangl2* RNA (Vangl2^{OE}) onto wild-type hosts. Ciliated cell orientation within these Vangl2^{OE} clones was also disrupted (Figure 3, compare [F] and [G]), varying significantly more around a mean direction compared to controls ($p = 0.0075$). Thus, both the loss- and gain-of-function experiments with Vangl2 support the idea that outer cells provide cues to orient ciliated cells and that the proper levels of Vangl2 are required to generate this cue.

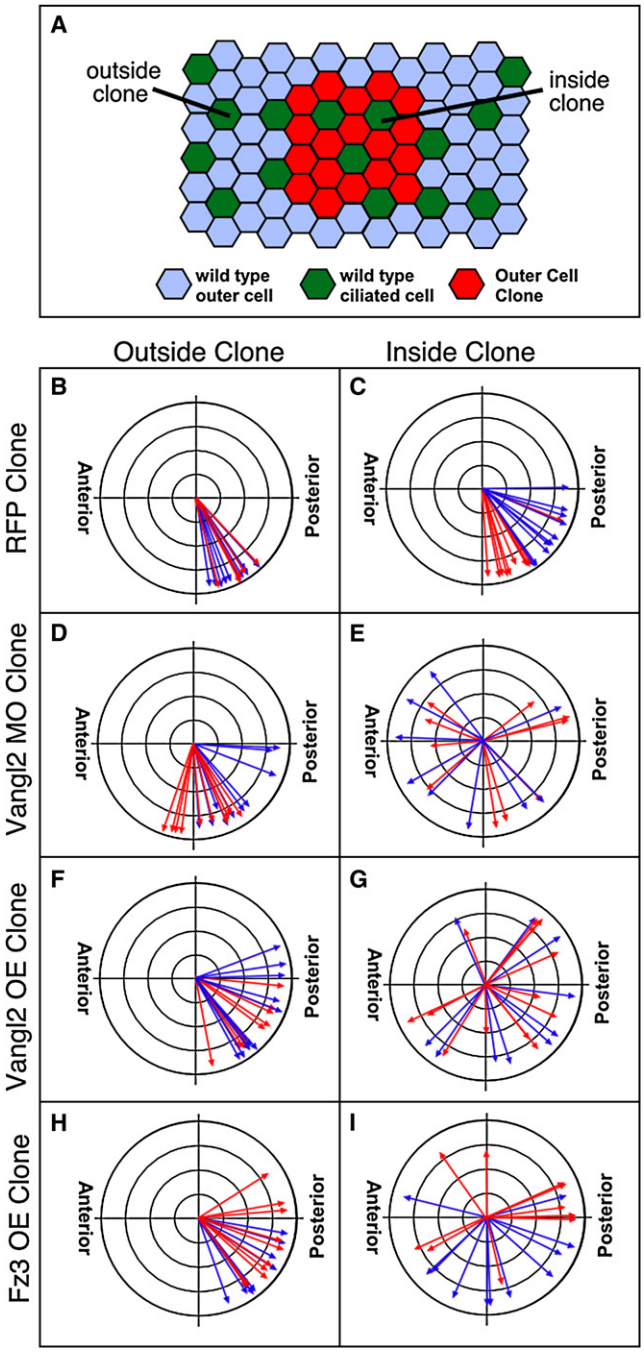

Figure 3. Ciliated Cell Orientation Is Disrupted Non-Cell-Autonomously by Changes in Vangl2 and Fz3 Activity

(A) Diagram of an assay in which outer cells from donor embryos injected with Vangl2^{MO}, *Vangl2*, *Fz3*, or *RFP* RNA were grafted onto wild-type host embryos. Ciliated cell orientation was measured with confocal microscopy (see Experimental Procedures and Figure 2) either outside of the clone (>30 cell diameters from the clone) or inside of the clone.

(B–I) Circular graphs of ciliated cell orientation for wild-type cells outside of the clones (B, D, F, and H) and for wild-type cells surrounded by transplanted outer cells injected with mRFP (D), Vangl2^{MO} (E), Vangl2 OE (G), or Fz3 OE (I).

In *Drosophila*, changing the activity of Frizzled in mutant clones also has profound noncell-autonomous effects on neighboring wild-type cells [1]. However, vertebrates have

a large number of Frizzled homologs, in contrast to Vangl2, and it is not clear which and how many of these might be required for PCP signaling in the skin [5, 12]. Therefore, we focused on the overexpression of Frizzled-3 ($Fz3^{OE}$) because mice mutant for Fz3, when combined with those in Fz6, have defects in axial elongation and polar orientation defects in hair cells [21]. When wild-type ciliated cells intercalate into outer cells that overexpress Fz3, their orientation is also disrupted, showing more variation around a mean direction compared to control (p = 0.00021) (Figure 3, compare [I] and [H]). Thus, these results suggest that changes in the levels of PCP signaling alter ciliated cell orientation non-cell-autonomously.

One interpretation of the results above is that outer cells require the proper levels of Vangl2 and Frizzled activity, perhaps indirectly, to generate an orientation cue for ciliated cells. Alternatively, Vangl2 and Frizzled may be acting as they do in PCP signaling in *Drosophila* by instructively polarizing neighboring ciliated cells. The key observations that distinguish between these two possibilities in *Drosophila* are the different directional nonautonomous phenotypes that occur at clone boundaries mutant for Frizzled and Van Gogh [7, 8]. Thus, to determine whether Vangl2 is also acting instructively in outer cells to orient ciliated cells, we analyzed the orientation of ciliated cells lying at the anterior and posterior boundary of an outer-cell transplant (Figure 4A and S4).

Ciliated cells located at the anterior and posterior borders of a clone of wild-type cells (mRFP) orient their cilia in the normal posterior direction with an ~45° ventral bias (Figures 4B, 4C, S5A, and S5B). Ciliated cells located at the anterior border of $Vangl2^{MO}$ clones are still oriented posteriorly but with a less pronounced ventral bias (Figures 4D and S5C). In stark contrast, ciliated cells at posterior border of $Vangl2^{MO}$ clones are significantly reversed, on average, 124° (p = 7.57E-5) relative to control cells and 166° relative to the cells at the anterior border (Figures 4E and S5D). We see a reciprocal effect on the orientation of ciliated cells at the border of $Vangl2^{OE}$ clones (Figures 4F and 4G). Ciliated cells at the anterior border of $Vangl2^{OE}$ clones (Figures 4F and S5E) are reversed 154° relative to controls (p = 1.81E-18), whereas those at the posterior border (Figures 4G and S5F) have lost their ventral bias and, thus, shifted 48° relative to the control. In *Drosophila*, Van Gogh and Frizzled have reciprocal effects on orienting cells at clone boundaries [6–8]. Though less striking than the Vangl2 results, the ciliated cells located at the anterior border of $Fz3^{OE}$ clones (Figure 4H) are shifted 37° relative to controls (p = 9.7E-7) but are still oriented in a posterior direction. Ciliated cells at the posterior borders, however, are reversed 71° relative to the controls in an anterior direction (p = 6.03E-7) and are, on average, shifted 108° relative to the cells at the anterior border. These results provide strong evidence that Vangl2 and Frizzled levels in outer cells instructively orient the planar polarity of ciliated cells but in opposite directions: ciliated cells orient the beating of their cilia toward outer cells with lower levels of Vangl2 activity and to those with higher levels of Frizzled.

These data support a model in which ciliated cells are patterned along the A-P axis of the developing skin based on cues that they encounter when they intercalate into the outer epithelium. Moreover, our data suggest that PCP signaling acts as a major cue in this patterning event whereby Vangl2 and Fz3 (or related Frizzled[s]) instruct the polar orientation of intercalating cells in a reciprocal manner. Therefore, the instructive signaling cues that we observe in our transplant assay provide strong functional evidence that ciliated cells

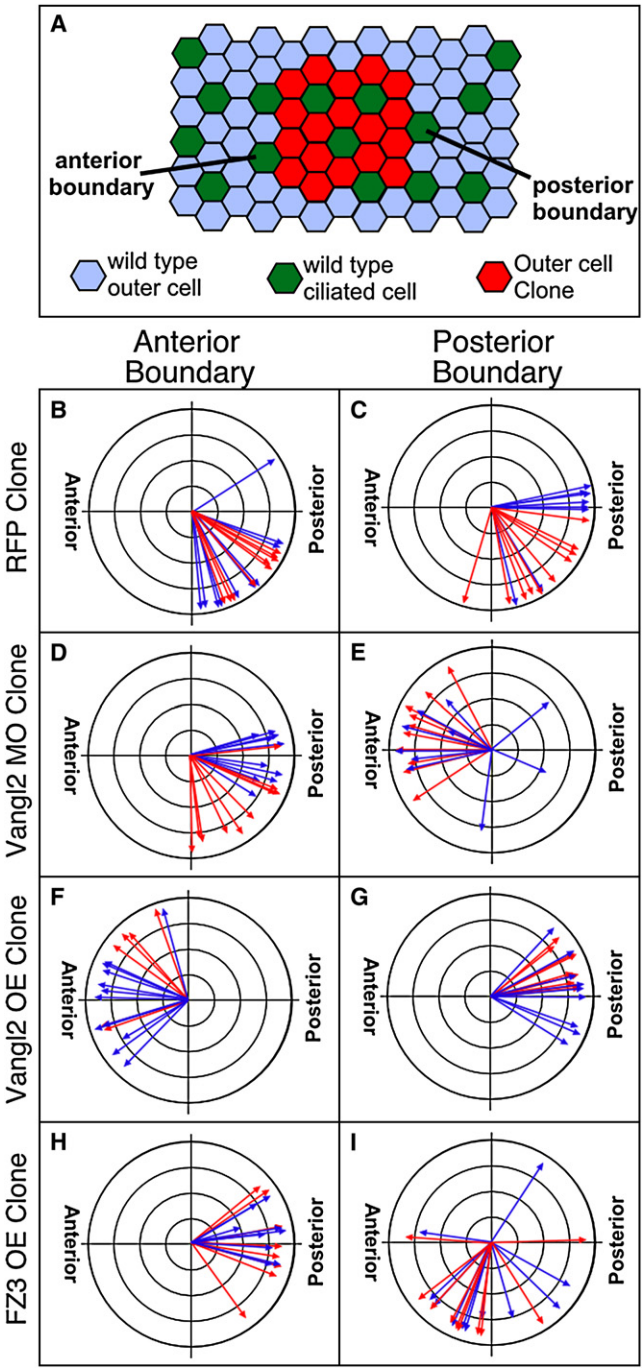

Figure 4. Vangl2 and Fz3 Have Directional, Non-Cell-Autonomous Effects on Ciliated Cell Orientation

(A) Diagram of an assay in which wild-type ciliated cells are located at the anterior and posterior border of an outer-cell clone from donor embryos injected with $Vangl2^{MO}$, *Vangl2*, *Fz3*, or *RFP* RNA. Ciliated cell orientation at the clone border was measured with confocal microscopy (Figure S4).

(B–I) Circular graphs of ciliated cell orientation for cells located at the anterior border (B, D, F, H) or for those located at the posterior border (C, E, G, I) under the indicated experimental conditions. Different colors represent data from different experiments.

are oriented by local asymmetry in PCP activity and represent the first direct evidence for directional noncell autonomy during PCP signaling in a vertebrate system. PCP signaling

in *Drosophila* is accompanied by and potentially attributed to the asymmetric localization of Frizzled and Van Gogh along the planar axis to opposite sides of each cell (reviewed in [4]). Accordingly, our functional data would predict that Vangl2 and Frizzled activity are differentially restricted to the posterior and anterior sides of skin cells, respectively. It remains to be determined whether this differential activity involves asymmetrical protein localization, as proposed in *Drosophila*, or another mechanism.

In contrast to Vangl2 and Fz3, the Dvl2 mutant Xdd1 did not cause a disorientation of ciliated cells when expressed in clones of outer cells, even though, in the converse experiment, it disrupted the orientation of BBs in a cell-autonomous manner. This result is reminiscent of findings in *Drosophila*, suggesting that Dishevelled is not required to generate or propagate intercellular PCP signaling, at least over clone distances, but is only required intracellularly for cells to polarize [22, 23]. Disrupting Dishevelled activity in the outer cells by using additional approaches will be required to fully address the role of Dishevelled in PCP signal propagation both intercellularly and intracellularly.

Our data also show that BBs fail to dock apically, and ciliogenesis fails in Vangl2 morphants, as reported previously for Dvl1-3, Inturned, and Fuzzy [13, 14]. Intriguingly, mammalian Vangl2 has been reported on vesicular structures that localize to the BB in human respiratory ciliated cells [24]. Our data, therefore, add further support to the idea that BBs are positioned apically in ciliated cells by vesicular targeting events involving multiple components of the PCP pathway [14]. Due to the ciliogenesis defects, we cannot test whether Vangl2 also has a cell-autonomous role in establishing the rotational orientation of individual BBs. Nonetheless, our observations suggest that, at the same time PCP components position BBs apically, they are also used to orient ciliated cells along the planar axis via interactions with cells in the outer epithelium.

Experimental Procedures

Transplant Assays and Explant Cultures

Xenopus laevis embryos were obtained by in vitro fertilization with standard protocols [25]. To mark transplanted tissue, we injected embryos four times at the two- to four-cell stages with capped, synthetic mRNA-encoding [25] membrane-localized form of RFP (mRFP). At stage 10, a fine needle or hair was used to peel off the outer layer from a region of the ectoderm from a donor embryo, which was transferred onto the host embryos after removing a similar patch of outer cells. While the transplanted tissue healed onto the host embryo, it was kept in place by pressing down with a small piece of a glass coverslip, held in place with silicone grease. In experimental transplants, host embryos were not only injected with mRFP but also with a Vangl2 MO (5′-ACTGGGAATCGTTGTCCATGTTTC-3′, Gene Tools), Control MO (5′-CTAGCGCTGTAAGGAGCCATCCTGT-3′), *Vangl2* [26], or *Fz3* RNA [27]. Transplants were performed in Danilchik's buffer + 0.1% BSA [28]. After healing of the transplanted tissue, we returned embryos to 0.1 × Marc's Modified Ringers (MMR) [25] until stage 28 when they fixed overnight on ice in 4.0% paraformaldehyde in phosphate-buffered saline (PBS). After mounting, tadpoles were imaged with a BioRad Radiance 2100 confocal mounted to a Zeiss inverted microscope with a 63× objective. Grafted tissues were identified based on the RFP tracer and were analyzed when localized to the middle flank. Ciliated cells were imaged that were either within the grafted tissues, at least 30 cell diameters outside the grafted tissue, or located at the anterior or posterior border and touching both grafted and host cells.

Confocal Assay for Cilia Orientation

To score ciliated cell orientation, we determined cilia direction along the polar axis by measuring BB orientation with confocal microscopy [14]. This assay involves expressing two fusion proteins in host embryos. One protein, called CLAMP, is fused to GFP and localizes to the striated rootlet, a structure that marks the rotational axis of the BB by projecting in the opposite direction of ciliary beating. The second protein, Centrin2, is fused to RFP and localizes to the basal body. When expressed in ciliated cells with RNA injection, the two fusion proteins decorate the BB and rootlet such that orientation can be easily scored by confocal microscopy. The orientation of ~100 BBs was scored per cell and used to calculate the mean orientation of cilia within a cell as a measure of a cell's overall planar polarity, wherein the mean direction of a cell is denoted as an arrow on a circular graph and the length of the arrow represents the variance around that mean [29].

Immunostaining and Confocal Microscopy

Ciliated cells were immunostained by fixing embryos with 4.0% paraformaldehyde in PBS for 1 hr on ice. Tissue was stained by overnight incubation in rabbit anti-ZO-1 (Zymed 1:200) and mouse monoclonal anti-acetylated tubulin (Sigma 1:1000) or anti-γ tubulin GTU88 (Sigma 1: 500) primary antibodies and by a 4 to 6 hr incubation in anti-rabbit Cy3 and anti-mouse Cy2 secondary antibodies (Jackson Immunoresearch). Antibody incubations were performed with PBS containing 0.1% Triton X-100 and 10% heat-inactivated normal goat serum and were washed with PBS containing 0.1% Triton X-100. Basal bodies and rootlets were labeled by injecting synthetic messenger RNA encoding centrin2-RFP and Clamp-GFP fused at the carboxyl terminus as previously described [14]. After mounting, embryos were imaged on a BioRad Radiance 2100 confocal mounted to a Zeiss inverted microscope with a 63× objective. Movies of cilia beating were taken at 6688 fps with a Vision Research Phantom 7.2 mounted to an Olympus BX51 microscope with a 100× objective.

Data Analysis

Basal body-rootlet orientation was scored with Matlab, and statistical analysis and circular plotting were done with Oriana 2.0 (Kovach Computing Services) circular statistics software. Each arrow on the polar plot represents the orientation of a single ciliated cell based on scoring the orientation of, on average, 100 cilia (basal body-rootlets) per cell. Experimental values were compared to control values with a two-tailed Student's t test.

Supplemental Data

Supplemental Data include five figures and five movies and can be found with this article online at http://www.cell.com/current-biology/supplemental/S0960-9822(09)00977-4.

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