Establishment of Global Patterns of Planar Polarity during Growth of the *Drosophila* Wing Epithelium

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Summary

Epithelial tissues develop planar polarity that is reflected in the global alignment of hairs and cilia with respect to the tissue axes. The planar cell polarity (PCP) proteins form asymmetric and polarized domains across epithelial junctions that are aligned locally between cells and orient these external structures [1–3]. Although feedback mechanisms can polarize PCP proteins intracellularly and locally align polarity between cells, how global PCP patterns are specified is not understood. It has been proposed that the graded distribution of a biasing factor could guide long-range PCP [4, 5]. However, we recently identified epithelial morphogenesis as a mechanism that can reorganize global PCP patterns; in the *Drosophila* pupal wing, oriented cell divisions and rearrangements reorient PCP from a margin-oriented pattern to one that points distally [6]. Here, we use quantitative image analysis to study how PCP patterns first emerge in the wing. PCP appears during larval growth and is spatially oriented through the activities of three organizer regions that control disc growth and patterning. Flattening morphogen gradients emanating from these regions does not reduce intracellular polarity but distorts growth and alters specific features of the PCP pattern. Thus, PCP may be guided by morphogenesis rather than morphogen gradients.

Results and Discussion

Global PCP Emerges during Growth and Aligns with Organizer Regions

To study the emergence of polarity in the wing disc, we quantified the subcellular distribution of the PCP proteins Flamingo (Fmi) and Prickle (Pk). We calculated planar cell polarity (PCP) nematics based on Fmi staining [6] and PCP vectors based on the perimeter intensity of EGFP::Pk clones (Figures 1A–1C; see also Figures S1A and S1B available online). At 72 hr after egg laying (hAEL), the wing pouch has just been specified and is small [7], EGFP::Pk localizes to punctate structures at the cell cortex that are asymmetrically distributed in some cells, but PCP vectors exhibit no long-range alignment (Figures S1D–S1G). By 96 hAEL, PCP vector magnitude increases (Figure S1C) and a global pattern emerges (Figures S1H–S1K). Later, PCP vector magnitude increases further (Figure S1C) and the same global polarity pattern is clearly apparent (Figures 1D–1F). It is oriented with respect to three signaling centers: the dorsal-ventral (DV) boundary (where Wingless [Wg] and Notch signaling occur), the anterior-posterior (AP) compartment boundary (where Hedgehog [Hh] and Decapentaplegic [Dpp] signaling occur), and with respect to the hinge fold (where levels of the atypical Cadherin Dachsous [Ds] change sharply).

PCP vectors in the wing pouch near the hinge fold point away from it toward the center of the pouch. Within the Wg expression domain at the DV boundary, PCP vectors parallel the DV boundary and point toward the AP boundary (Figures 1D–1F, red arrows, lines). Just outside this domain, PCP nematics and vectors turn sharply to point toward the DV boundary in central regions of the wing pouch. However, where the DV boundary intersects the hinge-pouch interface, they remain parallel to the DV boundary over larger distances such that PCP vectors orient away from the hinge around the entire perimeter of the wing pouch (Figures 1D–1F).

The AP boundary is associated with sharp reorientations of PCP. First, PCP vectors that parallel the DV boundary point toward the AP boundary in both anterior and posterior compartments (Figure 1E). Second, although PCP vectors in the central wing pouch are generally orthogonal to the DV boundary, they deflect toward the AP boundary where Hh signaling is most active (as defined by upregulation of the Hh receptor Patched [Ptc]) (Figures 1D–1F, blue lines, arrows, and Figure S1I). On either side of this region, PCP vectors turn sharply to realign parallel to the AP boundary. Third, PCP vectors in the hinge point away from the AP boundary and align parallel to the hinge fold (Figures 1D–1F, orange lines, arrows).

Fat and Dachsous Orient PCP near the Hinge

The atypical Cadherins Fat (Ft) and Ds limit disc growth and orient growth perpendicular to the hinge [8, 9]. Their loss perturbs the PCP pattern in pupal wings and alters hair polarity [10]. To investigate whether they influence the larval pattern, we quantified PCP in *ft* and *ds* mutant discs (Figures 2B–2D). The PCP pattern is similar to wild-type (WT) in the central wing pouch but altered in proximal regions close to the hinge fold (Figure 2). Polarity vectors deviate from their normal orientation (away from the hinge fold) in many regions of the proximal wing pouch. This is especially clear near the intersection of the DV boundary with the hinge—here, PCP vectors orient toward the DV boundary rather than away from the hinge (Figure 2; Figures S2A–S2A*). Furthermore, near the AP boundary, vectors form a reproducible point defect, with vectors pointing away from the defect center (Figure 2, red arrows in Figures S2B–S2C).

After pupariation, morphogenesis reshapes the wing disc, apposing its dorsal and ventral surfaces such that the DV boundary defines the margin of the wing blade (Figure S2F). During reshaping the PCP pattern evolves, but specific local features are retained through pupal development (Figures S2F–S2M). Consistent with this, hair polarity in *ds* adult wings reflects the perturbed larval pattern (Figure S2E); hairs in the

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proximal wing near the anterior wing margin orient toward the
margin rather than away from the hinge (Figures S2D and S2E).
Near the AP boundary, hairs form swirling patterns (Figure
S2E). Thus, Ft and Ds are required during larval growth
to ensure that PCP vectors in the proximal wing orient away
from the hinge.

The DV Boundary Organizer Orient PCP in the Central
Wing Pouch
Notch and Wg signaling at the DV boundary organize growth
and patterning in the developing wing. These pathways main-
tain each other via a positive feedback loop; Notch induces
transcription of Wg at the DV interface, and Wg signaling upreg-
ulates expression of the Notch ligands Delta (Dl) and Serrate
(Ser) adjacent to the Wg expression domain [11–15], further
activating Notch signaling at the DV boundary. To study how
the DV boundary organizer affects PCP, we ectopically ex-
pressed Ser along the AP boundary with ptc-Gal4 (ptc > Ser).
In the ventral compartment, Ser induces two adjacent
stripes of Wg expression, which then upregulate Dl expression in
flanking regions (dorsally, Fringe prevents Notch activation
by Ser [16]; Figure 3A; Figure S3D). The posterior Wg and DI
stripes are distinct, but the anterior stripes are broader due
to the graded activity of ptc-Gal4 (Figure 3A; Figure S3D).
In these discs, the ventral compartment overgrows along the
AP boundary, parallel to the ectopic “organizers” (Figure 3A).

PCP nematics and vectors near the posterior Wg/DI stripes
are organized similarly to those flanking the normal DV
boundary—running parallel to the stripe and turning sharply
outside this region to orient toward the ectopic organizer (Fig-
ure 3A; Figures S3C and S3C’). PCP nematics anterior to the
ectopic Ser stripe run parallel to it over larger distances before
turning sharply, consistent with the broader Wg/DI expression
in this region (Figure 3A). In resulting adult wings, hairs orient
toward the ectopic wing margin that forms along the AP
boundary (Figures S3A and S3B). Ectopically expressing Wg
along the AP boundary also generates an ectopic organizer
that reorients growth and PCP (Figure 3B).

To ask how loss of the DV boundary organizer affected PCP,
we used a temperature-sensitive allele of wg (wg<sup>TS</sup>) or
we populated wings with wg null mutant clones [17]. Loss of Wg signaling severs the feedback loop
with Notch such that both decay [14]. We quantified PCP
nematics in wg<sup>TS</sup> discs shifted to the restrictive temperature
shortly after the second to third-instar transition (earlier, Wg
is required to specify the wing pouch [18]). wg<sup>TS</sup> discs have
smaller wing pouches than WT and are missing a large fraction
of the central region of the pouch where polarity orients
perpendicular to the DV boundary (compare Figure 3C to 3H).
Polarity still orients away from the hinge, thus the PCP pattern
in wg<sup>TS</sup> discs appears more radial (i.e., oriented toward the
center of the wing pouch) (Figure 3C; Figures S3E–S3F’). Analog-
ously, adult wings populated by wg null clones are missing
those regions of the distal wing blade where hairs normally
point perpendicular to the wing margin (Figure 3E). The remain-
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Emergence of PCP in the Wing
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(ratio objects defined based on the perimeter intensity of PCP protein staining. They are used to characterize strength and orientation of the polarity axis in a single cell without specifying its vector direction. Nematics are visualized by lines. Line length represents the magnitude and line orientation represents the axis of polarity. They can be locally averaged to quantify polarity in groups of cells (see Supplemental Information).

(D) Cartoon illustrating the PCP pattern in a late third-instar wing disc.

(E) Averaged PCP vectors in WT (n = 9 discs, 1,801 clones), Region-specific color coding of averaged PCP nematics or averaged PCP vectors: green, ventral wing pouch; yellow, dorsal wing pouch; orange, hinge; red, Wg-expressing region; cyan, region of Ptc upregulation.

(F) Locally averaged PCP nematics from a Fmi stained disc overlaid on Wg + Ptc staining to indicate compartment boundaries. Scale bar represents 50 µm (see also Figure S1).
edges (Figures 3E and 3G). Here, polarity deflects from the proximal-distal axis to parallel the edge of the wing. Normally, hair polarity in the wing blade parallels the margin only in proximal regions, where Fts/Ds influences polarity (Figures 3D and 3F). Thus, the DV organizer is needed to orient PCP in distal regions perpendicular to the margin. Fts/Ds is required for a complementary subset of the PCP pattern in the proximal wing. Their influences largely reinforce each other (i.e., away from the hinge and toward the DV boundary or wing margin) except where the hinge and wing margin intersect. Here, loss of one signaling system expands the influence of the other.

Wg is distributed in a graded fashion and is a ligand for Frizzled (Fz) [19, 20]. Thus, it could bias the PCP pattern directly, e.g., by asymmetrically inhibiting interactions between Fz, Strabismus (Stbm), and Fmi or causing Fz internalization. If so, uniform Wg overexpression should prevent intracellular polarization or reduce cortical localization of PCP proteins. To investigate this, we overexpressed Wg uniformly (C765 > wg::HA; Figure S3I). Uniform Wg expression elongates the wing pouch parallel to the AP boundary (Figure 3I; Figure S3D'). Fmi and EGFP::Pk polarize robustly in these discs (Figure 3I; Figures S3H, S3J, and S3K); thus, the Wg gradient does not act directly on PCP proteins to induce or orient polarity. However, the pattern of PCP vectors and nematics is altered. PCP points away from the hinge (rather than perpendicular to the DV boundary) over larger distances compared to WT and then turns sharply to face the DV boundary in the middle of the wing pouch (compare Figure 3H to 3I). Because specific alterations in the PCP pattern are induced by uniform Wg overexpression, Wg protein distribution does not directly specify the new PCP pattern.

**Hh Signaling Interfaces Orient PCP near the AP Boundary**

To identify signals that influence the PCP pattern near the AP boundary, we examined the effects of uniform high-level expression of Dpp and Hh, two morphogens that form graded distributions near the AP boundary. Uniform Dpp expression does not influence the magnitude of PCP or the range over which PCP deflects toward the AP boundary (Figure 4A; Figure S4C). Interestingly, uniform Hh expression dramatically increases the range over which PCP deflects toward the AP boundary (Figure 4B; Figure S4D), suggesting that Hh is important for this aspect of the pattern. However it clearly indicates
that PCP vectors are not oriented directly by the graded distribution of Hh or by the graded activity of Hh signaling, because both are uniformly high in the anterior compartment of Hh overexpressing discs (Figure S4B). We therefore considered whether the apposition of cells with very different levels of Hh signaling might produce sharp bends in the PCP pattern. In WT discs, Hh signaling levels change at two interfaces: one along the AP boundary and one along a parallel line outside the region of highest Hh signaling where Ptc is upregulated. PCP vectors orient parallel to the AP boundary in the cells posterior to it, deflect toward the boundary anteriorly, and then reorient sharply outside of this region to align parallel to the AP boundary (Figures 1E, 1F, and 3H). Discs uniformly overexpressing Hh have only one signaling discontinuity (at the AP boundary), because Hh signaling is high throughout the anterior compartment (Figures S4A and S4B). This could explain why PCP in these discs remains deflected toward the AP boundary over longer distances.

To test this, we generated clones mutant for the Hh receptor Ptc, which constitutively activate signaling in the absence of ligand [21]. Quantifying PCP nematics in these discs reveals reproducible patterns of polarity reorientation at interfaces between WT and ptc− tissue. In WT tissue adjacent to ptc− clones, PCP aligns parallel to the clone interface. Due to the typical clone shape, this orientation is often consistent with the normal PCP pattern. However, PCP also aligns parallel to ptc− clones in regions where this is not so (Figure 4C; Figure S4F). Thus, ptc− clones exert a dominant effect on adjacent WT tissue. In contrast, on the mutant side of the clone interface, polarity tends to orient perpendicular to the interface (Figure 4C; Figure S4E). Thus, apposition of high and low levels of Hh signaling causes a sharp bend in the PCP pattern. Reorienting polarity by ptc− clones is also seen in adult wings (Figures 4D and 4D0). Thus, Hh signaling has two effects in WT discs: within the Hh signaling domain, it deflects PCP toward the AP boundary, and just outside the Hh signaling domain, it orients PCP parallel to the AP boundary. In this region, the tendency for polarity to align parallel to Hh signaling interfaces is consistent with the orientation of polarity toward the DV boundary and away from the

Figure 3. The DV Organizer Orients PCP in the Central Region of the Wing Pouch

(A) Locally averaged PCP nematics from a disc in which Ser expression is driven along the AP boundary under the control of ptc-Gal4 (ptc > Ser). PCP nematics are overlaid on Wg staining. Box indicates the region analogous to that shown in Figures S3C and S3C0.

(B) Locally averaged PCP nematics from a disc expressing HA-tagged Wg along the AP boundary under the control of dpp-Gal4 (dpp > wg::HA). PCP nematics are overlaid on Wg staining.

(C) Locally averaged PCP nematics in a wgTS disc overlaid on Wg and Ptc staining. Unsecreted Wg is still detected, but its expression is beginning to decay, confirming that Notch signaling is compromised.

(D–G) Hair polarity vectors calculated from tiled bright-field images of adult wings in WT (D) and ubx-Flip; wgC>FRT40 / Minute FRT40 (E). Adult wings consisting predominately of wg mutant tissue were generated by giving wg mutant cells a growth advantage using the Minute system. Red arrowheads indicate hair orientation. Boxed regions are enlarged in (F) and (G).

(F and G) Hair polarity at the distal posterior wing margin in WT (F) and ubx-Flip; wgC>FRT40 / Minute FRT40 (G).

(H) Locally averaged PCP nematics of a WT disc overlaid on Di staining. Di upregulation in longitudinal veins (L1, L3, L4, L5) is indicated.

(I) Locally averaged PCP nematics in C765 > wg::HA overlaid on Di staining. Boxed region corresponds to Figure S3J.

Locally averaged PCP nematics in (A–C), (H), and (I) based on Fmi staining. Scale bars represent 50 μm (A–C, F–I), and 500 μm (D and E) (see also Figure S3).
hinge. Thus, these three polarity cues reinforce each other throughout much of the wing pouch, making the global PCP pattern robust.

The PCP Pattern Is Not Directly Specified by Gradients

Simulations have highlighted the difficulty of establishing long-range polarity alignment in a large field of cells from an initially disordered arrangement [22, 23]. The pattern typically becomes trapped in local energy minima, forming swirling defects. Introducing a small bias in each cell removes such defects—this has been an attractive argument for the involvement of large-scale gradients in orienting PCP. The graded distribution of Ds along the proximal-distal axis (orthogonal to the hinge-pouch interface) suggested a plausible candidate for such a signal [24]. Strikingly, the Ds expression gradient gives rise to intracellular polarization of both Ft and Ds, and the recruitment of the atypical myosin Dachs to the distal side of each cell [25, 26]. Nevertheless, most of the PCP defects in ft mutants can be rescued by uniform overexpression of a truncated Ft version that cannot interact with Ds [27], and PCP defects in ds mutants can be rescued by uniform overexpression of Ds [6, 28, 29]. Moreover, blocking overgrowth through removal of dachs also suppresses PCP phenotypes in both mutants [25, 26]. The remaining disturbances in PCP in each of these backgrounds are restricted to very proximal regions, both in adult wings and the wing disc. Thus, the graded distribution of Ds does not provide a direct cue to orient PCP over long distances; rather, it appears to be important only locally near the hinge. Furthermore, we show here that the two other key signaling pathways that contribute to the global PCP pattern in the disc do not act directly through long-range gradients. How do these signals specify the PCP pattern, if not through gradients? Simulations in the vertex model have suggested that long-range polarity can be established in the absence of global biasing cues if PCP is allowed to develop during growth. PCP easily aligns in a small system, and globally aligned polarity can then be maintained as the system grows [6]. Such a model obviates the necessity of long-range biasing cues like gradients, at least to maintain long-range alignment of PCP domains. Our finding that a global PCP pattern develops early during growth of the wing makes this idea plausible. It may be that a combination of local signals at the different organizer regions specifies the vector orientation of PCP when the disc is still small, and that the pattern is maintained during growth. This may explain why loss-of-function studies have failed to identify the signaling pathways at the AP and DV boundaries as important organizers of the PCP pattern.

In addition to local signals, the orientation of growth may provide additional cues that help shape the PCP pattern. Simulating the interplay between PCP and growth in the vertex model showed that oriented cell divisions and cell rearrangements orient PCP either parallel or perpendicular to the axis of tissue elongation, depending on parameters [6]. Interestingly, each of the signaling pathways that influence PCP in the disc also influences the disc growth pattern. Wg/Notch signaling at the DV boundary drives growth parallel to the DV boundary (Figures 3A and 3B; [8, 30]), consistent with the pattern of clone elongation at the DV boundary [31]. Growth near the AP boundary, where Hh signaling is most active, is oriented parallel to the AP boundary [30]. This behavior probably reflects oriented cell rearrangements rather than oriented cell divisions [32]. Finally, Ft and Ds orient growth away from the hinge [8, 9]. Suppressing overgrowth in ft or ds mutant wings by altering downstream components of the Hippo pathway rescues normal PCP except in the most proximal regions of the wing [25, 26, 33]. Thus, altered growth orientation may contribute to the PCP defects seen in ft and ds mutants.

Growth orientation reflects the orientation of both cell divisions and neighbor exchanges, and these can each exert different effects on the axis of PCP [6]. Understanding the influence of local growth patterns on PCP will require a quantitative description of the patterns of cell divisions and rearrangements in the disc. More refined simulations incorporating local differences in the orientation of cell divisions and rearrangements will allow us to explore how planar polarity patterns can be guided by different growth patterns.

Experimental Procedures

A summary of fly lines, antibodies, and immunohistochemical protocols is provided in the Supplemental Information. Imaging of entire wing discs at cellular resolution was performed with an Olympus FV-1000 confocal microscope with a programmable stage (further details in the Supplemental Information).

Quantification of Polarity Vectors and Nematics

Cells were segmented based on E-Cadherin staining as described previously [6]. PCP nematics were calculated as described [6], except that the
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tensor components were normalized by the cell contour sum PCP intensity (see Supplemental Information).

PCP vectors $\vec{p}$ were computed individually for each EGFP:Pk clone (Figure 1B). They are defined as follows:

$$\vec{p} = \sum_{i} \mathbf{n}_i \mathbf{l}_i \mathbf{j}_i,$$

where the sums run over all cell boundaries $i$ between clonal cells and non-clonal cells. $\mathbf{n}_i$ is the unit vector normal to cell boundary $i$, pointing away from the clone. $\mathbf{j}_i$ is the sum of the pixel intensities of cell boundary $i$. The vector defined in Equation 1 points away from high Pk intensity and by implication toward high Fz intensity (Figure 1B).

To average polarity patterns over many discs, we used the compartment boundaries and the interface between the hinge and pouch to define a coordinate system for each disc quadrant. Clone position and vector polarity are mapped to an average wing disc using these coordinate systems (see Supplemental Information).

Quantification of Hair Polarity

Using bright-field images of adult wings, we calculated nematic from local anisotropies of pixel intensity correlations. This nematic provides an axis of local polarity throughout the wing. Manually specifying the vector direction within a small region, we iteratively determine the vector direction in the whole wing (see Supplemental Information).

Supplemental Information

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2012.04.066.

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