Wg and Wnt4 provide long-range directional input to planar cell polarity orientation in Drosophila

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Planar cell polarity (PCP) is cellular polarity within the plane of an epithelial tissue or organ. PCP is established through interactions of the core Frizzled (Fz)/PCP factors and, although their molecular interactions are beginning to be understood, the upstream input providing the directional bias and polarity axis remains unknown. Among core PCP genes, Fz is unique as it regulates PCP both cell-autonomously and non-autonomously, with its extracellular domain acting as a ligand for Van Gogh (Vang). We demonstrate in Drosophila melanogaster wings that Wg (Wingless) and dWnt4 (Drosophila Wnt homologue) provide instructive regulatory input for PCP axis determination, establishing polarity axes along their graded distribution and perpendicular to their expression domain borders. Loss-of-function studies reveal that Wg and dWnt4 act redundantly in PCP determination. They affect PCP by modulating the intercellular interaction between Fz and Vang, which is thought to be a key step in setting up initial polarity, thus providing directionality to the PCP process.

Planar cell polarity (PCP) is defined as cellular polarity within the plane of an epithelium and is associated with many tissues and organs. The conserved process of PCP establishment has been studied extensively in Drosophila, defining a core group of conserved factors around the Frizzled (Fz) protein, including the products of fz itself, Van Gogh (Vang; also known as strabismus/stbm), flamingo (fmi, also known as starry night/stan) and others. At early developmental stages, PCP core proteins interact with unknown upstream regulatory molecules as well as each other, to initiate and maintain their polarized localization, the first visible PCP feature. Within the Fz-core group, Fz is unique as it regulates PCP both cell autonomously and non-autonomously, with the extracellular domain of Fz acting as a ligand for Van Gogh (Vang; ref. 11). Fmi–Fz and Fz–Vang intercellular interactions are essential in specifying and propagating PCP direction. Fmi–Fmi interactions form homophilic bridges across cell membranes and facilitate intercellular Fz–Vang interactions, as Fmi physically interacts with both Fz and Vang. These factors also provide asymmetry to Fmi–Fmi interactions as FmI/Fz–Fmi/Vang intercellular bridges form homophilic bridges across cell membranes and facilitate intercellular Fz–Vang interactions, as Fmi physically interacts with both Fz and Vang. Although intracellular molecular events in Fz–PCP signalling are beginning to be understood, the mechanism(s) of long-range PCP orientation across whole tissues remain elusive. It has been proposed that graded Fz activity directs PCP orientation towards lower Fz activity, supported by manipulations of Fz levels. Endogenous Fz protein is, however, evenly expressed in all tissues tested, and therefore Fz ‘activity’ must be regulated post-translationally in a graded manner, the mechanism(s) of which are unknown.

Wnt proteins, mainly Wnt5a and Wnt11, are involved in vertebrate PCP establishment, but their mechanistic input to PCP regulation remains unclear. Although a Wnt5a gradient can generate a graded Vang2 phosphorylation during digit formation in mouse limbs, with this also depending indirectly on Ror2 activity, it is not resolved whether vertebrate Wnts provide a permissive or instructive input directly to regulate Fz–Fmi PCP interactions. PCP in Drosophila wing can be affected by morphogenetic organizers, with the dorsoventral organizer set up by Notch and Wg signalling and the anteroposterior organizer through Hedgehog and Dpp signalling in third instar larval discs. As the organizers control wing patterning, size and proliferation and the axes within the wing generally, their positional information could provide the earliest cues for PCP orientation. Consistent with our data shown later, PCP orientation of the vast majority of cells in the third instar larval wing pouch correlates with the dorsoventral organizer. Canonical Wg/β-catenin signalling serves a major patterning role at these early stages, and thus it is
Wnts are expressed throughout wing development and also in the eye PCP specification occurs at the stage when R3/R4 formation, APF), cellular PCP orientation is radial and polarity directed towards the margin (Fig. 1d–d”), where Wg and other Wnts are expressed throughout wing development (Fig. 1e), consistent with a hypothesis that Wnts could provide directional long-range control to Fz–PCP signalling. To circumvent potential redundancy associated with overlapping expression domains of several
Wnts (mentioned earlier), we first used gain-of-function (GOF) approaches by misexpressing each *Drosophila* Wnt in developing wings (using the Gal4/UAS system with either clonal or regional [dppGal4] expression)\(^{41}\). Whereas most Wnts did not show PCP effects (all Wnt genes in the genome were tested), expression of dWnt4 and Wg caused striking and classical PCP defects, affecting cellular (wing hair) orientation of neighbouring wild-type cells (Figs 2a–c and 3). These data are consistent with earlier reports, suggesting that misexpression of Wnt4 can cause PCP phenotypes in the wing\(^{42,45}\), and also with suggestions that Wg overexpression can affect PCP (ref. 31). Strikingly, the phenotypes of Wg and dWnt4 misexpression were similar to non-autonomous polarity defects associated with \(fz^-\) clones (surrounding wild-type cells orienting towards the mutant area\(^{9,24}\)) and opposite to \(fz\) GOF defects (wild-type cells orienting away from \(fz\)-overexpression regions\(^{9,11,24}\)).

To establish that these GOF phenotypes are mediated by \(fz\)–PCP signalling, and not by potential effects of the canonical Wnt/\(\beta\)-catenin pathway, we induced Wnt4-expressing clones in \(fz^-\) null mutant wings (the entire animal lacks \(fz\), but can signal canonically through the dFz2 receptor). Consistent with a direct effect of Wnt4 through \(fz\)–PCP signalling, the cellular reorientation of cells neighbouring Wnt4-expressing clones was eliminated in \(fz^-\) backgrounds (Fig. 2d) and cellular orientation was basically identical to the patterns in \(fz^-\) mutant wings alone (Fig. 2d,e; eight out of eight Wnt4-expressing clones caused no change to \(fz^-\) mutant wing cell orientations; see also Supplementary Fig. S1e,e′). Further confirming that the PCP effects were not mediated through canonical Wg/Wnt/\(\beta\)-catenin signalling, Wnt4-expressing clones did not affect expression of any canonical Wnt/Wg targets\(^{36-40}\) in larval or pupal wings (for example *Dll* and *pj-lacZ*, Supplementary Figs S1b–d). Taken together, the genetic epistasis data (Fig. 2d,e and Supplementary Figs S1e,e′) and the expression studies (Supplementary Figs S1b–d) indicate that the Wnt GOF PCP phenotypes are mediated through Fz–PCP signalling and that Fz acts downstream of Wnts in this context.

**Wnt misexpression alters Fz–PCP factor localization**

If Wnts regulate Fz–PCP activity, their misexpression should not only affect wing hair orientation, but also alter core PCP factor polarization and hence PCP axis orientation at critical early stages of PCP establishment. To examine this functionally, we used Wnt4 clonal expression (which has no effect on canonical Wnt signalling, mentioned earlier) and analysed pupal wings throughout PCP patterning. At early stages (prepupae–16 h APF), when PCP axes are radial\(^{17}\), Wnt4 clones strikingly reorient core PCP factor polarity (determined through Fmi staining) to a near 90° angle relative to clonal borders (Fig. 4a–a′,c,d–d′; compare with wild type, Fig. 4b,b′; see figure legend for statistical analyses). Similarly, at later stages (~30–32 h APF), when PCP orientation has been realigned to the proximodistal axis (and actin prehairs have started to form), Wnt4-expressing clones continue to reorient surrounding cells towards clonal borders (Figs 2c,c′ and 3b–b′,d; see legend for statistical evaluations). Of note, the near 90° reorientation of cells (relative to clonal borders, Fig. 4a,c,d) is mirrored by border cells on the inside of clonal margins, an effect probably mediated by intercellular feedback loops\(^{18,19}\). Also, Wnt4-expressing
cells inside clones are less polarized when compared with surrounding non-expressing cells as measured by their nematic order (length of polarity lines in Figs 3b” and 4a,d; see figure legend for statistics), the exception again being border cells (marked by orange dots in Fig. 4a”), which are polarized relative to their outside neighbours (blue dots in Fig. 4a”; see legend for details). Overall, the frequency (72%) and effects seen with the Wnt4-misexpression clones are very comparable to those with fz null clones (around 80% frequency in our hands), consistent with the notion that the Wnt effect and Fz–PCP-mediated orientation are linked. Taken together, our data indicate (1) that Wnt4 and Wg can reorient cell polarity towards their expression domain (similar to fz clones), and (2) that this polarity depends on a Wnt-expression border (and possibly graded Wnt distribution), rather than high uniform Wnt levels (as demonstrated by the reduction of nematic order/polarity strength inside Wnt-expressing clones; legend of Fig. 3). Both observations indicate that Wnts serve an instructive role in establishing a PCP
Figure 4 Wnt4 misexpression reorients PCP during early pupal development. (a–a'', d–d'') 16 h APF pupal wings with Wnt4-misexpression clones (marked by GFP, green; yellow shading in a'). (b,b') Wild-type control for direct comparison. The inset in b' illustrates the area shown in a,b (hinge region marked blue). Anti-Fmi (red in multicolour panels, monochrome in a',b') and anti-dPatj (blue in a,a',b') reflect cellular orientation (Fmi) and cell outlines (dPatj). Polarization (Fmi localization) was calculated/quantified as described37, illustrated as white or yellow lines in each cell: the orientation of lines indicates the polarity direction; the length indicates the strength of polarization/nematic order (see later). (a'') Cells bordering the Wnt4 source, on the inside and outside of the clone, are highlighted with orange and blue dots, respectively; see c for orientation angles. (c) Polarization of specific cell populations from a,b as labelled: i,ii, polarity relative to clonal border; iii–iv, actual polarity as present in wing area (n, number of cells scored/rosette; 36 Wnt4 GOF clones were analysed by Fmi staining, five independent wings were analysed at this stage (15–16 h APF) and 31 at later stages (see also Fig. 3)). Wnt4 misexpression affects cellular polarization autonomously (polarity distribution of Wnt4+ cells (iv) is different from wild type (wt, iii); $P = 10^{-7}$ with two-sample Kolmogorov–Smirnov test; Methods) and non-autonomously (polarities of neighbouring cells (two or three rows marked by blue dots in a'') are distinct from wild type in the same region (iii versus v, $P = 10^{-6}$ with Kolmogorov–Smirnov) and near 90° relative to clonal borders (ii). Border cells inside the clone (orange dots in a'') are also near 90° relative to the clonal border (compare ii and i) and distinct from wild type ($P = 10^{-5}$). (d–d'') Different example of a Wnt4+ clone at the same stage. Note the random polarity in the clone and near 90° orientation of border cells relative to the clonal border (blue dots in d, rosette in d''). Four out of five Wnt4-expressing clones analysed at 15–17 h APF altered Fmi polarization in neighbouring cells. Nematic order, represented by the length of polarity lines in each cell (a,b) is affected by Wnt-expressing clones. Wnt4+ cells have reduced polarity versus non-Wnt4-expressing (wild-type) cells (59.8 ± 13.2 versus 72.1 ± 15.8; $P = 0.005$ with Student $t$-test; average nematic order ± standard deviation is shown), whereas border cells (blue and orange dots in a') are more polarized (compared with Wnt4+ cells: 78.4 ± 15.2 versus 59.8 ± 13.2; $p = 0.004$; average nematic order ± standard deviation; n, number of cells; five independent wings were scored).
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Wg and dWnt4 are expressed in an identical pattern and show comparable PCP features (detected through Fmi staining; Figs 5a,5c). At the margin, Wg and dWnt4 are expressed at the boundary between wild-type and mutant tissue (Methods), most wings showed wild-type PCP features at the margin (Supplementary Fig. S2), accompanied by genetic wing margin defects37. In Wg CX3/CX14 mutants, wings that were shifted to the non-permissive temperature in early prepupa (Methods), most wings showed wild-type PCP features (Fig. 5a–a”). To further reduce Wnt function and to address potential redundancy between Wg and dWnt4, we examined double mutant wings for wnt4+/−, Wg CX3/CX14 (completely removing wnt4 and partially wg, shifted as above, Methods; importantly, the stronger wnt4 −/−, cx14 double homozygous animals were lethal before third instar stages even at the permissive temperature, hence the need for the hypomorphic wg CX3/CX14 combination). Strikingly, wings from such animals showed robust PCP defects, both in pupal wings (Fig. 5a–c”; note that such animals did not eclose and thus were analysed at pupal stages with molecular markers) and at early stages (prepupa −16 h APF; Fig. 6). PCP defects were indicated not only by misoriented cellular hairs (actin staining in Fig. 5b, green; see also Supplementary Fig. S3e–f for orientation defects in rare adult escapers), but importantly also by defects in the early pupal polarization axes of core PCP factors (detected through Fmi staining; Figs 5a–c and 6). Importantly, these results suggest a requirement of wg, dWnt4 at the critical, early stages of PCP axis definition. Whereas wild-type cells polarize towards the wing margin and show strong polarization (measured by nematic order, length of yellow lines in individual cells in Fig. 6a,b,c; longer lines correspond to stronger polarization), the wg, dWnt4 double mutant wings lacked coordinated cellular orientation; mutant cells showed a wide distribution of polarity angles (Fig. 6b–c”) and showed reduced overall polarization (evident in nematic order levels reduced to 60%, P = 10−28, in the double mutant; see Fig. 6 legend for details). Interestingly, cells near the wing margin were oriented parallel to the margin in the double mutant background (for example pink ‘nematic order’ lines in Fig. 6b”, which is consistent with the proposal that cells in/at the margin respond to cues from the anteroposterior organizer (in third instar stages)31 and are less polarized in wild type at early pupal stages37 (pink ‘nematic order’ lines in cells in Fig. 6a,c and ref. 37). Together with the GOF data, these results indicate that Wnts are not only instructive but also necessary for Drosophila PCP axis establishment.

Wg and dWnt4 are necessary for PCP establishment

To determine whether Wnt4 and/or Wg are also necessary for Fz–PCP axis orientation, we examined loss-of-function (LOF) phenotypes. As Wg and dWnt4 are expressed in an identical pattern and show comparable GOF PCP effects (mentioned earlier), the lack of a LOF phenotype in dWnt4 EMS23 genetic null mutants (Fig. 5d; dWnt4 mutants also do not show PCP phenotypes in the eye35) could be due to redundancy with wg. LOF wg wings show strong PCP defects throughout development, precluding simple PCP analyses. To limit the early Wg requirements, we used a temperature sensitive allele, wg EMS23 / ts at the permissive temperature, hence the need for the hypomorphic wg CX3/CX14 combination. Strikingly, wings from such animals showed robust PCP defects, both in pupal wings (Fig. 5a–c”; note that such animals did not eclose and thus were analysed at pupal stages with molecular markers) and at early stages (prepupa −16 h APF; Fig. 6). PCP defects were indicated not only by misoriented cellular hairs (actin staining in Fig. 5b, green; see also Supplementary Fig. S3e–f for orientation defects in rare adult escapers), but importantly also by defects in the early pupal polarization axes of core PCP factors (detected through Fmi staining; Figs 5a–c and 6). Importantly, these results suggest a requirement of wg, dWnt4 at the critical, early stages of PCP axis definition. Whereas wild-type cells polarize towards the wing margin and show strong polarization (measured by nematic order, length of yellow lines in individual cells in Fig. 6a,b,c; longer lines correspond to stronger polarization), the wg, dWnt4 double mutant wings lacked coordinated cellular orientation; mutant cells showed a wide distribution of polarity angles (Fig. 6b–c”) and showed reduced overall polarization (evident in nematic order levels reduced to 60%, P = 10−28, in the double mutant; see Fig. 6 legend for details). Interestingly, cells near the wing margin were oriented parallel to the margin in the double mutant background (for example pink ‘nematic order’ lines in Fig. 6b”, which is consistent with the proposal that cells in/at the margin respond to cues from the anteroposterior organizer (in third instar stages)31 and are less polarized in wild type at early pupal stages37 (pink ‘nematic order’ lines in cells in Fig. 6a,c and ref. 37). Together with the GOF data, these results indicate that Wnts are not only instructive but also necessary for Drosophila PCP axis establishment.

Wg/Wnt4 modulate intercellular Fz–Vang interaction

Fz and Vang/Stbm interact physically with each other through their extracellular domains between cell membranes of neighbouring cells in culture and in vitro11,15 and thus presumably also in vivo. This interaction is thought to serve as a sensing mechanism of Fz levels/activity, and thus a determinant of PCP direction11. As Wnts bind Fz cysteine-rich domains50–52 and affect PCP orientation (Figs 2–6), we hypothesized that a Wnt–Fz association could modulate the Fz–Vang interaction, thus creating the ‘sought-after’ Fz–PCP activity gradient; it would be oriented along a Wnt gradient emanating from the source of Wg/Wnt4, as suggested by our GOF and LOF studies. We first tested whether Wg and/or Wnt4 can interfere with the Fz–Vang interaction in a cell-based assay15. This assay is well suited, as it directly detects an intercellular Fz–Vang interaction across cell membranes of neighbouring cells, similar to the context of PCP axis specification. Fz-expressing S2 + R cells recruit Vang in neighbouring cells to the membrane through cell–cell contact (Fig. 7a,b). As S2 + R cells are normally not adhesive and only a very small fraction of Fz- and Vang-expressing cells touch each other, we aided adhesion by co-transfecting with Drosophila E-cadherin (E-cad; to promote cell–cell contacts through homophilic E-cad interactions) with either Fz or Vang. Fz/E-cad-expressing cells recruit Vang to the membrane at cell contacts with Vang/E-cad-expressing cells (Fig. 7a–a”). To test whether Wg or Wnt4 can affect this Fz–Vang recruitment, we co-transfected the Fz/E-cad cells with plasmin encoding Wg. Strikingly, co-transfection of Wg reduced Vang recruitment in a specific and dosage dependent manner (Fig. 7c–e and see also Supplementary Fig. S4a; quantification reflects Vang membrane localization in individual cells in contact with Fz-expressing cells; control transfections included non-related secreted ligands, for example the Jak/STAT-signalling ligand Upd; Fig. 7c–c”). To confirm that this is a non-autonomous (extracellular) effect, we tested conditioned media (CM) containing secreted Wg or Wnt4 (and again Upd as control) in the same assay. The Fz–Vang recruitment was similarly decreased in Wg-CM and Wnt4-CM conditions (Fig. 7f and again Upd as control) in the same assay. The Fz–Vang recruitment was similarly decreased in Wg-CM and Wnt4-CM conditions (Fig. 7f and Supplementary Fig. S5a). These data confirm that Wg/Wnt4 can act as dosage dependent modulators of the Fz–Vang interaction during PCP establishment. To confirm these effects on Fz activity in vivo, we tested whether the Fz GOF PCP phenotype can be modified by Wnt4 co-expression. Strikingly, co-expression of Wnt4 with Fz (in the dpp–Gal4 domain11) markedly reduced the non-autonomous effects of Fz overexpression on cells outside the dpp domain (Fig. 8a–c and Supplementary Fig. S5a). These data are consistent with a previous report showing that Wnt4 can antagonize Fz-overexpressing phenotypes32 (although a different Fz phenotype was assessed there32). In summary, Wnt4/Wg can modulate the Fz–Vang interaction, possibly by antagonizing Fz signalling activity to neighbouring cells.

DISCUSSION

Our data indicate that Wg/dWnt4 regulate the establishment of Fz–PCP axes by modulating the Fz–Vang intercellular interactions in a graded, dosage dependent manner. Consequently they might generate different levels of Fz–Vang interactions across a Wg/dWnt4 gradient experienced by cells (see the model in Fig. 8d). This process is reiterated across the tissue, and the directionality of Fz–Vang binding is subsequently reinforced by intracellular core PCP factor interactions18,19. Our data are consistent with a model in which Wg/dWnt4 generate a Fz ‘activity’ gradient by modulating its capacity to bind to Vang, consistent with...
proposals of Fz ‘activity’ gradient models. Accordingly, PCP axes are orientated towards the Wg/dWnt4 source, which is evident in (at least) the wing and eye. The early wing PCP axis (late larval to early pupal stages) correlates well with Wg/dWnt4 margin expression and, similarly, in the eye polarity is oriented in the dorsoventral axis towards the poles where Wg/Wnt4 are expressed (illustrated in Supplementary Fig. S1a–a’). This model, relying on a Fz–Vang interaction, is also compatible with the addition of Fmi to this scenario, with intercellular (homophilic) Fmi–Fmi interactions also being required for PCP specification. As Fmi forms complexes with both Fz and Vang, the full complement of intercellular interactions includes Fz/Fmi–Fmi/Vang complexes, and these interactions would also be modulated by Wnt binding to Fz, either directly as proposed in our model or possibly by modulating the Fmi–Fmi interactions by Fmi being associated with Fz that is bound to different levels of Wg/Wnt4. In vivo, Fmi helps to enrich both Fz and Vang to the subapical junctional region, and Fmi–Fmi interactions bring Fz and Vang to close molecular proximity. Intercellular Fmi–Fmi interactions are strong, as Fmi-expressing S2 cells form cell aggregates through homophilic Fmi interactions. The interaction between Fz and Vang is weaker, and cell–cell contacts between the two cell groups are infrequent. It was suggested that PCP signal sensing complexes include both Fmi and Fz on one cell interacting with Fmi/Vang at the surface of a neighbouring cell. Within these complexes, Fz is required for sending a polarity signal, whereas Fmi and Vang are involved in its reception, consistent with our data and model. Although it has been suggested that Fmi is capable of sensing Fz/Fmi signals in the absence of Vang, the ‘Fz-sensing’ capability of cells with Fmi alone (lacking Vang) is much weaker.

Figure 5 dWnt4, wg double mutant LOF wings show PCP defects. (a–c) Pupal wings; d, an adult wing; anterior is up and distal to the right. (a–a”) wnt4EM23/wgCX3, single mutant) pupal wing, shifted from 16.5 to 29°C at 0–1 h APF and incubated for 29 h before dissection. Fmi (red; monochrome in a’) and phalloidin (green, showing actin prehairs) staining is shown. Most such wings show a normal PCP appearance, comparable to wild type. Cellular polarity (and nematic order), as highlighted by Fmi and, an adult wing; anterior is up and distal to the right. (a”, b”) wnt4−/−, wgCX2/16 double mutant pupal wing, shifted and stained as in a (same wing region as in a). PCP defects are evident by misoriented Fmi staining/polarity orientation (red; monochrome in a’) and wing hair orientation (phalloidin, green). Cellular polarity (yellow lines in b’) was calculated as in a’ and resulting polarity distribution is shown in b” (n, number of cells; five wings were analysed). It is distinct from that of a control (a,a’) wing in the same area (P = 10−22 with two-sample Kolmogorov–Smirnov test; Methods). Nine out of 10 wnt4−/−, wgCX2/16 double mutant wings show such Fmi localization PCP defects, whereas wnt4−/− (wnt4EM23) wings did not show PCP defects (d). (c,c’) A further example of a wnt4−/−, wgCX2/16 double mutant pupal wing for comparison.
Wnt LOF clones of Df(2L)NL Wg/Wnt4 requirement not observed before? Previous work attempted wing margin loss pointed towards remaining wing margin areas, the weak polarization in these cells; and PCP orientation changes from wings shifted at 01 h APF to 29° (APF at 25°). All panels show 16 h APF pupal wings (anterior is up and distal to the right), with the respective polarity distributions in rosette diagrams. (a-a''') Wild-type (wt), and b-c'', wnt4−/−, wg^{C53/18} double-mutant pupal wings shifted at 0–1 h APF to 29°C and incubated for 15 h (about 16 h APF at 25°C). (a',b') High-magnification view of boxed areas in a and b. (c) The same pupal wing area of an independent wnt4−/−, wg^{C53/18} double mutant. Fmi staining (monochrome) was used as a PCP orientation marker and polarity and strength/nematic order were plotted as yellow lines (a'b'b'c'). Cells close to the wing margin are less (or not) polarized, as previously reported (the three margin proximal rows highlighted by pink lines were excluded from polarity analysis in rosette diagrams in a'' and b''; for comparison; all other rosette diagrams include than that of cells with Vang^{17}. It will be interesting to determine if there are other PCP regulators directly involved in modifying Fmi–Fmi interactions.

How do our data relate to previous models and why was the Wg/Wnt4 requirement not observed before? Previous work attempted to address the role for the wing margin in PCP by examining either mutants affecting wing margin cells without eliminating wg/Wnt expression^{33} or in clones^{33}. Although cellular hairs near the site of wing margin loss pointed towards remaining wing margin areas, the effect was considered weak. We also examined potential effects of Wnt LOF clones of Df(2L)NL, lacking wnt4, wg, wnt6 and wnt10. In contrast to the global reduction of Wg/Wnt4 through the temperature sensitive wg allele, such clones cause only mild PCP perturbations (Supplementary Fig. S3a–c). There are several reasons why clonal loss of Wnt expression in the margin only mildly affects PCP orientation: cells can respond to Wnts from several sources/cells from remaining Wnt-expressing wing margin regions (illustrated in Supplementary Fig. S3a); polarization strengths (measured by nematic order) in the first few rows of cells near the margin are much weaker than those in cells further away (at 14–17 h APF; ref. 37) and weak PCP reorientation in cells neighbouring wing margin clones could thus reflect the initial weak polarization in these cells; and PCP orientation changes from all cells. (d) The sum of three independent wnt4−/−, wg^{C53/18} mutant wings, wnt4−/−, wg^{C53/18} double-mutant wings showed much wider polarity distribution than wild-type (compare a''' with b''' (P = 0.002 with two-sample Kolmogorov-Smirnov test, n, number of cells); or a''''''', d (P < 10−6); three out of three wnt4−/−, wg^{C53/18} wing discs quantified showed very similar phenotypes; two examples are shown in b and c; d shows the sum of all three). The overall polarization/nematic order level was markedly reduced from wild type to the wnt4−/−, wg^{C53/18} double mutants (wt, 59.13±36.65, versus mutant, 35.68±20.00, p = 10−12 with t-test; average nematic order ± s.d.). All double mutant wings analysed show complete lack of orientation and reduced overall polarization (three out of three quantified wnt4−/−, wg^{C53/18}; all others not quantified in detail showed visually very similar phenotypes).

To determine the direct role for Wg/Wnts on Fz–PCP signalling, we examined it at pupal stages, as the patterning role for canonical Wg signalling is much reduced then and PCP still correlates well with Wg/Wnt4 expression (ref. 37 and this study). Importantly, Wnt4 does not affect expression of patterning genes through canonical signalling at larval or pupal stages (Supplementary Fig. S1), yet Wnt4 alters PCP orientation, consistent with the model that Wnt4/Wg act directly on Fz–PCP interactions. The observation that Wnt4 requires Fz to affect neighbouring cells (Fig. 2d,e) further supports this model. It is likely that, as well as the Wg/Wnt4 input and mechanism identified here, both early and late PCP axes depend on further cues, provided for instance by the parallel Ft/Ds–PCP system or other morphogenetic.

Figure 6 dWnt4, wg double mutants show early loss of PCP orientation. All panels show 16 h APF pupal wings (anterior is up and distal to the right), with the respective polarity distributions in rosette diagrams. (a-a'''') Wild-type (wt), and b-c'', wnt4−/−, wg^{C53/18} double-mutant pupal wings shifted at 0–1 h APF to 29°C and incubated for 15 h (about 16 h APF at 25°C). (a',b') High-magnification view of boxed areas in a and b. (c) The same pupal wing area of an independent wnt4−/−, wg^{C53/18} double mutant. Fmi staining (monochrome) was used as a PCP orientation marker and polarity and strength/nematic order were plotted as yellow lines (a'b'b'c'). Cells close to the wing margin are less (or not) polarized, as previously reported (the three margin proximal rows highlighted by pink lines were excluded from polarity analysis in rosette diagrams in a'' and b''; for comparison; all other rosette diagrams include than that of cells with Vang^{17}. It will be interesting to determine if there are other PCP regulators directly involved in modifying Fmi–Fmi interactions.

How do our data relate to previous models and why was the Wg/Wnt4 requirement not observed before? Previous work attempted to address the role for the wing margin in PCP by examining either mutants affecting wing margin cells without eliminating wg/Wnt expression^{33} or in clones^{33}. Although cellular hairs near the site of wing margin loss pointed towards remaining wing margin areas, the effect was considered weak. We also examined potential effects of Wnt LOF clones of Df(2L)NL, lacking wnt4, wg, wnt6 and wnt10. In contrast to the global reduction of Wg/Wnt4 through the temperature sensitive wg allele, such clones cause only mild PCP perturbations (Supplementary Fig. S3a–c). There are several reasons why clonal loss of Wnt expression in the margin only mildly affects PCP orientation: cells can respond to Wnts from several sources/cells from remaining Wnt-expressing wing margin regions (illustrated in Supplementary Fig. S3a); polarization strengths (measured by nematic order) in the first few rows of cells near the margin are much weaker than those in cells further away (at 14–17 h APF; ref. 37) and weak PCP reorientation in cells neighbouring wing margin clones could thus reflect the initial weak polarization in these cells; and PCP orientation changes from all cells. (d) The sum of three independent wnt4−/−, wg^{C53/18} mutant wings, wnt4−/−, wg^{C53/18} double-mutant wings showed much wider polarity distribution than wild-type (compare a''' with b''' (P = 0.002 with two-sample Kolmogorov-Smirnov test, n, number of cells); or a''''''', d (P < 10−6); three out of three wnt4−/−, wg^{C53/18} wing discs quantified showed very similar phenotypes; two examples are shown in b and c; d shows the sum of all three). The overall polarization/nematic order level was markedly reduced from wild type to the wnt4−/−, wg^{C53/18} double mutants (wt, 59.13±36.65, versus mutant, 35.68±20.00, p = 10−12 with t-test; average nematic order ± s.d.). All double mutant wings analysed show complete lack of orientation and reduced overall polarization (three out of three quantified wnt4−/−, wg^{C53/18}; all others not quantified in detail showed visually very similar phenotypes).
Figure 7 Wg and Wnt4 inhibit intercellular Fz recruitment of Vang to the cell membrane. (a–a′) S2 + R cells co-transfected with plasmids encoding Fz-YFP (yellow fluorescent protein) (green) and DE-cad were mixed with S2 + R cells co-transfected with plasmids encoding Vang-Flag (red) and DE-cad. Vang (red) is recruited to cell membrane (arrow) in areas that are in contact with neighbouring Fz-expressing cells. Anti-GFP and anti-Flag were used to detect Fz-YFP and Vang-Flag proteins, respectively. (b) Vang-Flag, E-cad-transfected cells alone (not mixed with Fz-transfected cells) show Vang protein in vesicular structures, but not at the membrane. (c–c′′′) S2 + R cells co-transfected with plasmids encoding Fz-YFP (green), DE-cad (blue) and Unpaired (Upd) were mixed with S2+ cells co-transfected with plasmids encoding Vang-Flag (red) and DE-cad (blue). Upd was used as a control secreted ligand; it did not affect intercellular Vang recruitment by Fz (arrow). Cells in contact expressed DE-cad localized to their contact sites (blue; monochrome in c′′′). (d–d′′′) S2 + R cells co-transfected with plasmids encoding Fz-YFP (green), DE-cad (blue) and Wg were mixed with S2+ cells co-transfected with plasmids encoding Vang-Flag (red) and DE-cad (blue). In the presence of Wg (0.8 µg), Fz largely failed to recruit Vang to the contacting cell membranes. Fz membrane localization was not affected. (e) Quantification of Wg effect on intercellular Fz–Vang recruitment. Fz/DE-cad cells were co-transfected with increasing amounts of Wg DNA (as indicated), and subsequently mixed with Vang/DE-cad-transfected cells. Vang recruitment shown as percentage of total cell pairs analysed (n, number of contacting pairs; three independent experiments were used to score these). Statistical analysis: Fischer's exact test (two tailed) and P values are as indicated. The inhibitory effect of Wg on Fz–Vang recruitment is dosage dependent and highly reproducible (see Supplementary Fig. S4 for further examples). (f) Secreted extracellular Wnt4 and Wg affect Fz–Vang interaction. CM with Wnt4 or Wg affect Fz–Vang recruitment; Fz/DE-cad- and Vang/DE-cad-transfected S2 + R cells were incubated in Wnt4, Wg or Upd-V5 (as negative control) CM. Recruitment was quantified as percentage of Fz/DE-cad and Vang/DE-cad cell–cell contacts resulting in Vang localization at contact membranes. Vang recruitment was reduced in the presence of Wnt4 or Wg CM; n, number of contacting cell pairs (statistical analysis as in e; confirmed by three independent experiments; see Supplementary Fig. S4 for experimental samples).
ARTICLES

Figure 8 In vivo co-expression of Wnt4 inhibits non-autonomous effects of Fz. (a–c) Adult wings of indicated genotypes, expressing the indicated transgenes under dppGal4 control (expression domain marked by transparent orange staining); distal is right. Orientation of neighbouring (wild-type) cells is indicated with green arrows. With dppGal4, UAS-Wnt4 alone the transgene has largely a wild-type appearance (a), whereas dppGal4, UAS-Fz (with UAS-fac2+ as control) causes robust reorientation of neighbouring cells (b); aberrant orientation highlighted with green arrows. Co-expression of Fz with Wnt4 (dppGal4, UAS-Fz/UAS-Wnt4) markedly reduces the non-autonomous effects of Fz (c). See Supplementary Fig. S5 for quantitative and statistical analyses. (d) Model: schematic illustration of the molecular mechanism involving Wg/Wnt4 in modulating Fz activity for the Fz–Vang interaction: and associated PCP direction establishment. Wg/Wnt4 binding to Fz generates a “Fz–PCP activity” gradient for the Fz–Vang interaction. See main text for details and other potential interpretations.

METHODS

Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.W. and M.M. planned the project, designed and carried out the experiments, analysed the data and wrote the paper; A.R. and J.C. analysed the data and designed quantitative analytical tools for the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Fly strains. UAS-wnt4, UAS-wg, UAS-Fz, wnt4; wg; Df(2L)NL were as described previously; wnt4; wg; Df(2L)NL double mutant chromosomes were obtained through standard meiotic recombination using as markers markers carrying mobile elements M1 and M2 respectively. M1 and M2 flies were obtained from the Bloomington Drosophila Stock Center (stock number 23180 and 18023). M1 wnt4; wg were then recombined to obtain M1 wnt4; wg. M1 and M2 flies were used as described previously. M1 wnt4; wg M2 CyO flies was confirmed by genetic crosses (M1 wnt4, M2 CyO genomic DNA and sequenced to confirm the presence of the wnt4, M2 CyO genomic DNA). M1 and M2 were out-crossed from M1 wnt4; wg and M1 wnt4; CyO flies. We generated M1 wnt4; wg, M2 CyO flies by a very similar strategy. wnt4; M2 CyO flies don’t survive to late third instar stages even at 16°C (permissive temperature for wnt4; M2 CyO flies). Genotypes used in this paper. (1) Wnt4-expressing clones: hs-flp, act > > Gal4, UAS-GFP/++; UAS-wnt4 (M13)/+. Clones were induced during first and second instars, then kept at 29°C. (2) Wnt4-expressing clones in F2/F1 wing: hs-flp, act > > Gal4, UAS-GFP/++; UAS-wnt4 (M13)actp1/F1. Clones were induced during first and second instars, then kept at 29°C. (3) Wg Expression during prepupal/pupal stages: tub-Gal80; dpp-Gal4/UAS-wg, kept at 18°C, then shifted to 29°C. (4) Wnt4 mutant: wnt4; M2 CyO, kept at 16°C, then shifted to 29°C. (5) wg mutant: wg; M2, kept at 16.5°C, then shifted to 29°C. (6) Wnt4 double mutant: M1 wnt4; M2/M2 CyO, kept at 16.5°C, then shifted to 29°C. (7) Comparing Fz gain of function and Fz/Wnt4 gain of function (a) dpp-Gal4/UAS-wnt4 (M13) (b) UAS-lacZ1/+; dpp-Gal4, UAS-Fz/ + (c) dpp-Gal4, UAS-Fz/UAS-wnt4 (M13)

Immunohistochemistry and immunocytochemistry. The following antibodies were used:

- Anti-Wg, anti-Fmi and anti-DE-cad antibodies were obtained from the Developmental Studies Hybridoma Bank (DSHB) at the University of Iowa: anti-Wg, DSHB; clone 4D4 anti-Fmi, ref. 12; DSHB, clone 74 anti-DE-cad, DSHB, clone DCAD2 anti-dPAtj, ref. 63, non-commercial anti-Dll, ref. 64, non-commercial mouse anti-Flag, Sigma, catalogue No. F1804, clone M2 chicken anti-Flag, Aves, catalogue No. GFP-1020 rabbit anti-GFP, Invitrogen, catalogue No. A-11122 goat anti-mouse IgG, Alex A668: Invitrogen, catalogue No. A-11031 donkey anti-rabbit IgG, FITC: Jackson ImmunoResearch catalogue No. 711-095-152 donkey anti-chicken IgY, FITC: Jackson ImmunoResearch catalogue No. 703-095-155 goat anti-rabbit IgG, Alexa 647: Invitrogen catalogue No. A-21245 goat anti-rat IgG, Alexa 647: Invitrogen catalogue No. A-21247 donkey anti-rat IgG, TRITC: Jackson ImmunoResearch catalogue No. 722-025-153 donkey anti-rat IgG, Cy5: Jackson ImmunoResearch catalogue No. 712-175-153 goat anti-rabbit IgG, DyLight 649: Jackson ImmunoResearch catalogue No. 111-495-144, discontinued.

- Donkey anti-rat IgG, DyLight 649: Jackson ImmunoResearch catalogue No. 722-495-153, discontinued.

- Anti-Wg, anti-dPAtj, anti-DE and anti-Fmi antibodies were used at 1:20, 1:1000, 1:500 and 1:10 dilutions, respectively. Chicken or rabbit anti-GFP was used at 1:10,000–50,000 (Aves) or 1:10,000–20,000 (Invitrogen). FITC–phalloidin and rhodamine–phallodin were used at 1:500–1000 (Invitrogen). Mouse anti-Flag (M2 clone) was used at 1:250–500 (Sigma). Rat anti-DE-cad was used at 1:20–30 (DSHB). S2-R cells were fixed in 2% formaldehyde in PBS for 40 min and washed once with PBS. Cells were permeated in PBS 0.1% Triton X-100 for 8–10’, rinsed once in PBS, then incubated in PBS 0.1% BSA for 10–20’. Primary antibodies were added in PBS 0.1% BSA and incubated with cells for 2 h. Cells were then washed in PBS three times and in PBS 0.1% BSA once. Fluorescent secondary antibodies were added in PBS 0.1% BSA and incubated with cells for about 1 h. Cells were then washed four times in PBS, and mounted onto slides in 80% glycerol 0.4% n-propyl gallate mounting medium.

- Larval and pupal wing discs were dissected and fixed in PBS 4% formaldehyde 0.1% Triton X-100 for 20 min (larval discs) or 1-3 h (pupal wings). Tissues were washed twice in PBS 0.1% Triton X-100 (PBST) and incubated in 0.1% BSA for 15-30 min. Primary antibodies were added to tissues and incubated overnight at room temperature. Samples were washed three times in PBST and once in PBST 0.1% BSA. Fluorescent secondary antibodies were added in PBST 0.1% BSA and incubated for 2 h. Samples were washed four times in PBST, rinsed once in PBS and mounted on slides in 80% glycerol 0.4% n-propyl gallate mounting medium.

Fz–Vang recruitment assay. Effectene (Qiagen) was used to transfect DNA plasmids into S2 + R cells following the general transfection protocol. The Fz–Vang intercellular recruitment assay was carried out by mixing two populations of cells transfected with different DNA constructs. The first population of cells was transfected with 0.6 µg pAC-Fz–GFP, 0.6 µg pAC–DE-cad with either 0.8 µg pAC–upd–V5, a mix of 0.6 µg pAC–upd–V5 and 0.2 µg pAC–wg, or 0.8 µg pAC–wg in six-well plates. The second population was transfected with 0.6 µg pAC–Vang–Flag, 0.6 µg pAC–DE-cad and 0.8 µg pAC vector in six-well plates. Cells were incubated with Effectene/DNA mixtures in Schneider cell culture media with 10% fetal bovine serum for about 4–6 h. They were then collected and spun down, and cell pellets were washed twice through resuspension and centrifugation with 3 ml Schneider cell culture media, then resuspended in 1 ml media with 10% fetal bovine serum. The two populations of cells were subsequently mixed and cultured for 2 days on coverslips coated with 0.1 mg/ml 1% poly-l-lysine in 12-well plates. Conditioned media of Upd-V5, Wnt4 and Wg were collected from S2 + R cells transfected with Upd-V5, Wnt4 and Wg plasmids, then cultured for 3–5 days. These cells for the recruitment assay were fixed and stained with anti-GFP (detecting Fz–GFP), anti-Flag (detecting Vang–Flag) and anti-DE-cad (detailed protocol available on request). In this recruitment assay, only Vang/DE-cad-expressing cells in contact with Fz/DE-cad-expressing cells are assayed for Vang membrane recruitment. The recruitment percentage reflects the ratio between the number of Vang-recruiting cell pairs and the total number of Fz/DE-cad and Vang/DE-cad contacting pairs. Non-contacting cells are not included in the analysis. Fisher’s exact test (two tailed) was used to analyse statistical significance in Vang recruitment between different test samples.

Quantitative analyses of hair orientation and cellular polarity vectors. A suite of several MATLAB programming scripts was designed and developed to automatically analyse microscopy photographs. A picture was read, processed by contrast and histogram filtering, and segmented to detect each object in the figure. Subsequently, the morphological properties of these objects were extracted to retrieve unique ‘hairs’ in the photograph along with their specific features such as orientation, size or polarity. In addition, extensions of this algorithm enabled us to automatically or manually select regions of interest inside each photograph and analyse series of pictures or fluorescence microscopy photographs, among others. Rosette figures were generated to represent the 360° orientation of the cellular population, with 0° always being oriented as pointing distally in pupal or adult wings. The MATLAB function designed to obtain the distribution of cellular orientations from Drosophila pupal wings or adult wing pictures can be downloaded from http://www.neural-circuits.org/other-software/hairorientation. The script probably needs to be modified depending on the type of image, but could be used for other related polarity features.

- Statistical tests, using the two-sample Kolmogorov–Smirnov test, were used to assess differences between cellular orientation distributions of independent individual samples that can contain non-independent cells. In general, two-sample Kolmogorov–Smirnov tests compare two data sets obtained from two independent samples. Within these independent samples, individual data points do not need to be independent, as is often the case in PCP studies. As such, we generally used the formula to compare two non-independent data sets of two independent samples, such as wild type versus mutant.

- Polarity as determined from anti-Fmi stained cells was calculated with the software ‘packing_analyzer_V2’ as described in18. The software calculated both angles and strength of polarization (nematic order)27. Nematic order is calculated as the strength (extent) of polarization and angle is calculated as the direction of polarization within a cell. Both measurements are based on the staining intensity around the cell membrane. The detailed method and mathematical formulae used in the calculations are described in ref. 57. Images were analysed without adjustment of contrast and brightness to enable the most accurate representation of cellular polarity and strength of polarity. The resulting polarity ‘angle lines’ were
then processed using the MATLAB software described above for rosette-diagram presentations and statistical analyses.

The sample size was chosen by phenotypic features to represent a field of cells as large as possible in any given experiment. Each such ‘sample field’ was compared from several independent experiments as outlined above. No statistical method was used to predetermine sample size. Each sample size was determined by the size of the mutant clone analysed. No animals were excluded from samples if they were of the correct genotype. The mutant samples were selected randomly as they were found in the animals. The investigators were not blinded to allocation during experiments and outcome assessment.

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Figure S1 Wnt4 PCP phenotypes are not mediated through canonical Wnt signaling. (a-a') Illustration of wg, dWnt4 and dWnt6 expression patterns in wing (a) and eye discs (a'), relative to the proposed Fz activity gradient (arrows indicate direction of PCP). (b-b') A late 3rd instar larval wing disc containing Wnt4 expressing clones (labeled by GFP, green) and stained for fj-lacZ (blue) and anti-Dll (red) expression, both defining canonical Wnt/b-catenin signaling targets. Note that their expression is not affected by Wnt4 misexpression. Panel b' and b'' show individual channels of fj-lacZ and Dll staining. These data demonstrate that canonical Wnt/b-catenin signaling is not affected by Wnt4 misexpression. Panel b' and b'' show individual channels of fj-lacZ and Dll staining. These data demonstrate that canonical Wnt/b-catenin signaling is not affected by Wnt4 misexpression. (c-c') 30-35h APF pupal wing with ectopic Wnt4 expressing clones (marked by GFP, green) and stained for fj-lacZ (blue, single channel in C'). fj-lacZ expression is not affected by Wnt4 expressing clones. Dll is also not affected, expressed high at the wing margin and low flat expression across wing blade. Wnt4 clones were induced during first and early second larval instar. (d) Adult wing containing an Arm510 (stabilized active form of b-catenin) expressing clone (labeled by GFP co-expression). Arm510 represents activated canonical Wg-signaling. Although Arm510 expressing cells induced ectopic anterior wing margin fate (as expected for high level canonical Wg-signaling), indicated by margin bristles and vein like appearance, these cells did not alter the cellular polarity of neighboring cells. Clones were induced during the second larval instar. These data document that the gain-of-function PCP effects of dWnt4 (or Wg) are not mediated via canonical Wnt-signaling and thus likely through direct effects on Fz/PCP signaling. (e, e') Two examples of ectopic Wnt4 expressing clones (green) in fzP21 mutant wings. Ectopic Wnt4 expression did not alter PCP (as determined by cellular hair orientation) of surrounding cells in fzP21 mutant background.
Figure S2  Reduction of Wg expression at the wing margin in \(wg^{CX3}\) clones. (a-a') \(wg^{CX3}\) mutant clones labeled by lack of b-gal staining (green; white lines indicate clonal borders in a'). Anti-Wg is in red (monochrome in a'). Clone at left (yellow arrow) affects Wg expression domain on both sides of D/V boundary with Wg expression levels reduced in mutant area (yellow area), compare to adjacent wild-type tissues. Red arrow marks Wg levels in a clone that affects only expression in dorsal compartment; Wg is again reduced but to a lesser extent (red arrow), as Wg expressed on ventral side can diffuse into (dorsal) area.
Figure S3 Planar cell polarity phenotypes in wg, dWnt4 adult escaper animals. (a-c) PCP phenotypes associated with wnt4-, wg-, wnt6-, wnt10- LOF clones. Red area in (a) indicates region shown in (b) and (c). (a') Schematic illustration how cells (green area) can receive Wnt signals from two sides of remaining wing margin, when part of the wing margin is lost due to either wg clones or other manipulations. (b) Wild-type wing: cells orient towards wing margin as indicated by cellular hairs (summarized by red arrows). (c) Wings containing Df(2L)NL mutant clones (Df(2L)NL removes dWnt4, wg, dWnt6 and dWnt10; dWnt4, wg, and dWnt6 are all expressed at the wing margin). Loss of wg in wing margin cells causes loss of margin bristles, a high-threshold target of canonical Wg-signaling and thus loss of margin bristles serves as a marker for mutant clones in margin cells. Arrows indicate cellular orientation of cells surrounding wild-type area (displaying wing margin fate), which express the Wnt genes. Areas with no margin are mutant and have no local Wnt expression. Note that cells orient locally towards the patches of wild-type margin (Wnt-expressing cells), and parallel to areas that have mutant (missing) “margin”, consistent with the notion that cells orient towards Wg/Wnt expression domains. (d-f) wnt4-/-, wgCX3/IL114 double mutant phenotypes in wings of rare adult escapers. Red box in panel (d) indicates area of interest in panels (e) and (f). Wings are oriented such that vein 2 is at 0°. (b-b') In wild-type wings cellular hairs are oriented along wing vein 2 (near 0°). (b-b') In wild-type wings cellular hairs are often pointing away from wing margin (green arrows). Polarity distribution quantification reveals that wing hairs orient at minus 15-45° in the double mutant background (p=10^-6 as compared to wild type; n= number of cells; statistical analysis was performed with the Kolmogorov-Smirnov test designed to compare two independent populations/patterns of cells).
Figure S4 Wg and Wnt4 affect intercellular Fz-Vang recruitment in S2+R cells. (a) Quantification of Wg effect on intercellular Fz-Vang recruitment, note dosage dependent inhibition of Vang membrane stabilization event (see also Fig. 7 and main text). Increasing amounts of Wg DNA (as indicated) were co-transfected with Fz/DE-cad cells, which were subsequently mixed with Vang/DE-cad transfected cells. The inhibitory effect of Wg on Fz-Vang recruitment is dosage dependent and highly reproducible (n=number of contacting cell pairs between Fz/DE-cad expressing cells and Vang/DE-cad expressing cells). Statistical analysis: Fischer’s exact test (two-tailed) and p values are as indicated (n=number of cell pairs in contact). (b-d) Examples of Fz-Vang recruitment in the presence of conditioned media (CM) of Upd-V5 (b-b′), note normal Vang stabilization (red in b,b′) at contact membranes; Wnt4 (c-c′′), note loss of Vang at cell membranes; or Wg (d-d′′). Whereas Fz-Vang membrane recruitment is not affected in the presence of Upd CM (b), it is largely lost from cell contacts when cells were exposed to Wnt4 (c) or Wg (d) CM.
Figure S5  Quantification of Wnt4 effect on non-autonomous Fz gain of function in vivo. (a) Illustration of area of interest for quantification (example in wild-type wing). All wings are oriented anterior up and distal to the right (photographed with 20X objectives at 1280 pixels X 1024 pixels: grey box is about 550-600 pixels away from margin). Low edge of grey area is at the middle between vein 3 and 4, reflecting border of dpp-expression domain in pupal wings. Green box (600 pixels X 70 pixels), includes about 4 rows of cells adjacent to dpp-expression domain, is quantified in panels (b-d). (b-d) Examples of wings of the indicated genotypes: (b) dppGal4/UAS-Wnt4, (c) UAS-LacZ/+; dppGal4, UAS-Fz, (d) dppGal4, UAS-Fz/UAS-Wnt4. Green boxes are drawn as in a. (b’-d’) Orientation angle distributions (5 wings were quantified for each genotype). Co-expression of Wnt4 with Fz reduced the “pointing away” orientation from the Fz overexpression domain as compared to control (c’ vs d’; p<10^-14; n=numbers of cellular wing hairs [equivalent to cells]; statistical analysis was performed with the Kolmogorov-Smirnov test designed to compare two independent populations/patterns of cells.