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The Ankyrin Repeat Protein Diego Mediates Frizzled-Dependent Planar Polarization

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Summary

During planar polarization of the *Drosophila* wing epithelium, the homophilic adhesion molecule Flamingo localizes to proximal/distal cell boundaries in response to Frizzled signaling; perturbing Frizzled signaling alters Flamingo distribution, many cell diameters distant, by a mechanism that is not well understood. This work identifies a tissue polarity gene, *diego,* that comprises six ankyrin repeats and colocalizes with Flamingo at proximal/distal boundaries. Diego is specifically required for polarized accumulation of Flamingo and drives ectopic clustering of Flamingo when overexpressed. Our data suggest that Frizzled acts through Diego to promote local clustering of Flamingo, and that clustering of Diego and Flamingo in one cell nonautonomously propagates to others.

Introduction

Many types of epithelial cells polarize the localization of subcellular components, not only along their apical basal axis, but along an axis in the plane of the epithelium. Planar, or tissue, polarization is critical for the formation of well-organized tissues (Eaton, 1997). For example, the polarity of cilia constructed by oviduct epithelial cells allows them to all beat in the same direction. The polarization of stereocilia bundles on sensory hair cells maximizes the shear forces that lead to membrane depolarization. In insects, the uniform orientation of cuticular hairs and bristles reflects the underlying planar polarization of epidermal epithelial cells.

The planar polarization of *Drosophila* wing epithelial cells is apparent in the uniform distal orientation of wing hairs. Each cell constructs one hair by extending an actin and microtubule-filled process from its apical membrane toward its distal neighbor (Wong and Adler, 1993; Eaton et al., 1996). Hair formation is presaged by focused actin polymerization at a single distal site on the apico-lateral cortex and by the accumulation of microtubules that run from the center of the cell toward this cortical site. Genetic screens in *Drosophila* have identified a set of "tissue polarity genes" that mediate planar polarization of epithelia. The products of tissue polarity genes specify the placement and coherence of this distal cortical site and the subsequent orientation of hair outgrowth, although they are not required for hair formation itself (Wong and Adler, 1993).

Intriguingly, a "core" group of tissue polarity genes mediates planar polarization of more than one type of tissue; *frizzled*, *prickle/spiney legs*, *dishevelled*, and *strabismus (Van Gogh)* appear to be components of a signaling pathway that polarizes not only wing hairs, but also bristles and the orientation and rotation of ommatidial clusters in the eye (Krasnow et al., 1995; Zheng et al., 1995; Tomlinson et al., 1997; Taylor et al., 1998; Wolff and Rubin, 1998; Gubb et al., 1999). In the eye, planar polarity is established in each ommatidial cluster when two of the epithelial cells that will give rise to the six outer photoreceptors become asymmetrically rearranged (Mlodzik, 1999; Reifegerste and Moses, 1999). In wild type, this rearrangement occurs with opposite chirality in the dorsal and ventral halves of each eye. At the same time, the ommatidal clusters actively rotate toward the dorso-ventral midline, which forms a line of mirror-image symmetry. The mechanism by which these cellular rearrangements occur is not well understood, but it does not share obvious features with the processes that mediate hair outgrowth. This suggests that the products of these genes organize a very basic type of polarity that can be interpreted by different cell types in different ways.

Frizzled, a seven-pass transmembrane protein, appears to be a component of a complex signal transduction pathway that communicates the axis of planar polarity from cell to cell. Loss of Frizzled signaling in clones of cells within the wing induces polarity defects not only in mutant tissue, but also in wild-type tissue many cells distant (Adler et al., 1997; Vinson and Adler, 1987).

Other tissue polarity genes function mainly cell autonomously, and their products may be involved in organizing polarity within cells, or between cells and their immediate neighbors, in response to Frizzled signaling. One such gene is Flamingo. Flamingo (also known as Starry night) is a seven-pass transmembrane protein with Cadherin repeats and homophilic binding activity (Chae et al., 1999; Usui et al., 1999). It localizes to proximal and distal boundaries of wing epithelial cells in response to signaling by Frizzled (Usui et al., 1999), but the mechanism by which it does so is unknown. Still less is understood about how Flamingo, present on both proximal and distal sides of the cell, can determine the polarity of the planar axis and the mechanism by which this is translated into polarized cytoskeletal rearrangements.

Because most known tissue polarity genes also cause polarity defects when overexpressed, we reasoned that an overexpression screen might efficiently identify novel

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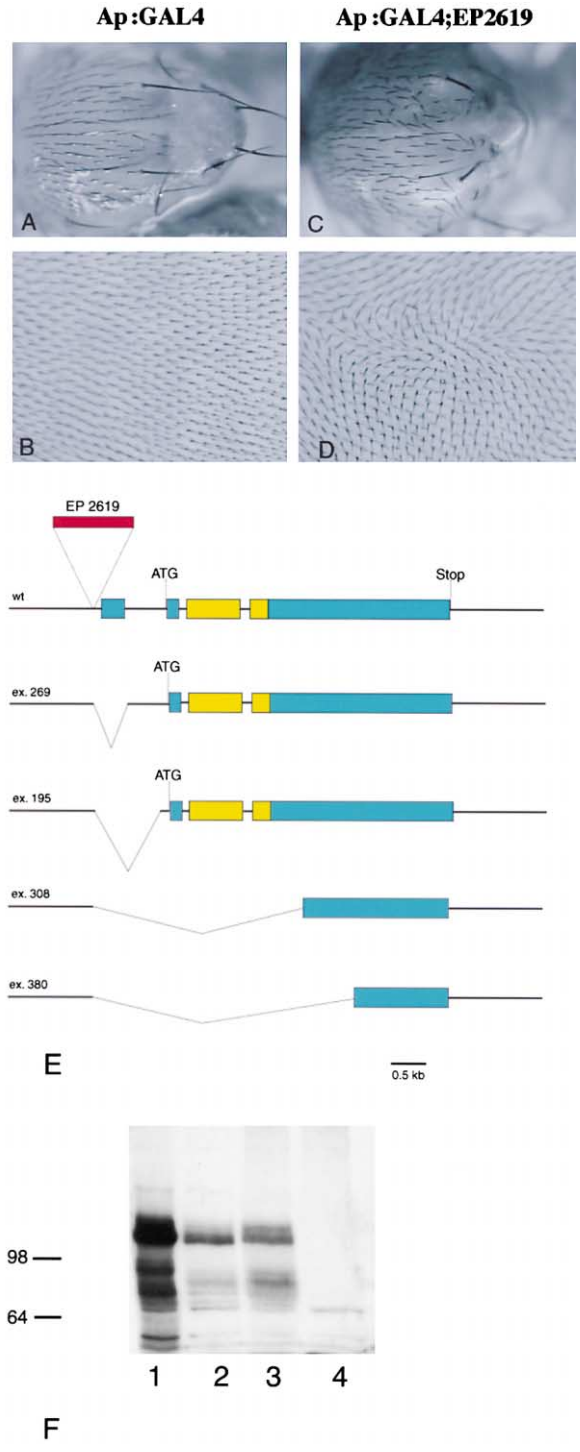


Figure 1. Expression from EP(2)2619 Causes Tissue Polarity Defects in the Thorax and Wing

(A) Thorax of *Ap:*GAL4/CyO.

(B) Wing of *Ap*:GAL4/CyO.

(C) Thorax of *Ap*:GAL4/CyO; EP(2)2619.

(D) Wing of *Ap:*GAL/CyO;EP(2)2619.

(E) Map of the Diego genomic region and approximate location of the end points of excision mutations. Yellow indicates the regions containing the six ankyrin repeats. The end points of deletions 269 and 195 were determined by estimation of the size of PCR fragments spanning the deletions. The end points of 308 and 380 were additionally mapped by sequencing of PCR products.

components of the polarization mechanism. In this study, we describe a core group tissue polarity gene, *diego,* identified using this method.

Results

To isolate genes whose overexpression caused tissue polarity defects, we crossed lines harboring "EP" P elements (Rorth, 1996) to flies expressing GAL4 under the control of the *apterous* promoter. EP elements contain upstream activation sequences that drive GAL4-dependent transcription from an adjacent P element promoter through genomic DNA next to the site of insertion. Activation of transcription by GAL4 in *apterous*-expressing cells results in the overexpression of random genomic sequences in cells that give rise to the thorax and dorsal wing blade. We screened through 2300 EP crosses for those that produced bristle polarity defects in the thorax (33/2300), and then we reexamined these EP lines for their ability to cause defects in hair polarity or number. We found six that produced multiple wing hairs when overexpressed, but we found only one, EP(2)2619, whose overexpression caused predominantly hair polarity defects (Figures 1A–1D). We cloned the DNA downstream of the EP element by plasmid rescue, sequenced it, and found that it was homologous to an EST corresponding to clone LD08259 (CG12342 in the GadFly database).

To ask whether expression of the gene represented by this EST actually caused the polarity phenotype, we produced transgenic flies containing the cDNA under the control of the UAS promoter and drove its expression by crossing them to flies that expressed GAL4 under the control of the apterous promoter. Expression of LD08259 caused polarity defects similar to those observed when EP 2619 was crossed to *apterous* GAL4 (data not shown). These data confirm that the protein encoded by LD08259 causes tissue polarity defects when overexpressed. We refer to the gene represented by this cDNA as *diego*.

The sequence of the *diego* cDNA predicts a 106 kDa protein containing six ankyrin repeats at its N terminus. It differs in the first 22 amino acids from that of the protein predicted from the genome sequence (AAF58749 in the Gadfly database), but it is otherwise in agreement. An error report for the gene-encoding protein AAF58749 has been submitted to Flybase. To see whether Diego was homologous to a protein of known function, we searched available databases for related sequences with BLAST. Its closest relative is KIAA0957, a human cDNA of unknown function derived from a brain library, with which it shares sequence both inside and outside of the ankyrin repeat regions. Conversely, the sequence most closely related to KIAA0957 is *diego,* suggesting that the genes may be orthologues.

(F) Western blot of protein from (1) 35 *Ptc:*GAL4; UAS:*dgo* third instar wing discs, (2) 65 wild-type third instar wing discs, (3) 32 wild-type pupal wings, and (4) 32 *dgo380* pupal wings. The blot was probed with anti-Diego antibody.

Loss of *diego* Function Produces Polarity Defects in Wings and Eyes

In order to determine whether loss of *diego* function caused tissue polarity defects, we generated *diego* mutants by imprecise excision of EP 2619. We identified six mutants harboring deletions that removed parts of the *diego* transcript, but left sequences upstream of the P element intact (Figure 1E). These *diego* mutants are homozygous viable with wing hair polarity defects that resemble those of the strongest viable *flamingo/starry night* allelic combinations (Figure 2A). Bristle polarity was not strongly affected by *dgo* mutation (data not shown).

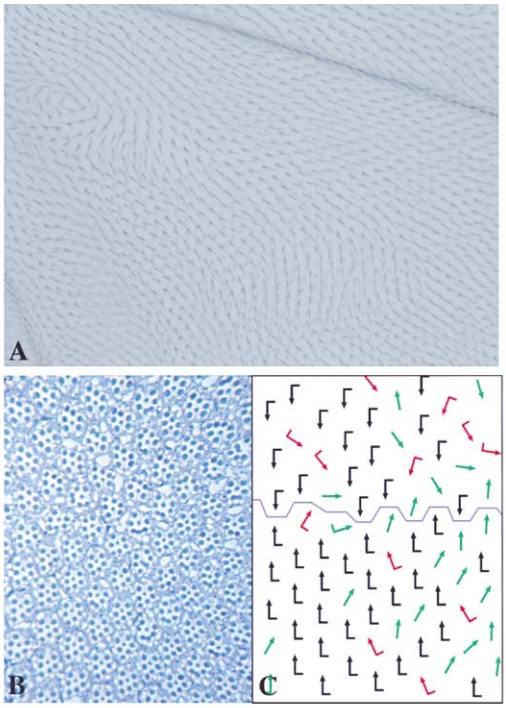
Diego mutant eyes also display typical planar polarity defects (Figures 2B and 2C); some ommatidia have inverted chirality, others are symmetrical, whereas others are misrotated. These defects are summarized in Figure 2D. The *diego* mutant phenotype is reminiscent of that observed for other "core" polarity genes like *fz* (Tomlinson et al., 1997). Of the *diego* mutant alleles, *dgo 380* (which harbors the largest deletion) produces the strongest eye phenotypes, and its phenotype is not stronger over a deficiency, indicating that it is probably a null allele. Consistent with this, no Diego protein is detected on Western blots of *dgo 380* homozygotes (Figure 1F). All *dgo* alleles appear to have similar wing phenotypes that are not stronger when hemizygous. This suggests that whereas all the *diego* alleles are at least strong hypomorphs for wing-specific functions, the shorter deletions may retain some ability to function in the eye. The fact that Diego is required for planar polarization of both eye and wing epithelia places it in the "core" group of tissue polarity genes and suggests that it forms an integral part of the polarization machinery.

Diego Protein Localizes to Proximal-Distal Boundaries of Wing Epithelial Cells in Response to Frizzled

The activity of the Frizzled signaling pathway is required during the six hours prior to hair formation for normal planar polarization (Adler et al., 1994). To better understand the cell biological function of Diego protein during this time, we generated antibodies to a region COOH-terminal to the ankyrin repeats. This antibody recognized a band of the expected size on Western blots of wild-type pupal wings that was of similar mobility to the protein produced by GAL4-driven overexpression and was undetectable in the *Dgo380* mutant (Figure 1B).

Immunofluorescence analysis of pupal wings at 18 hr showed that Diego localized in a spotty pattern all around apico-lateral junctions, with no apparent polarity (Figure 3A). By 24 hr, however, Diego became localized to proximal and/or distal cell boundaries (Figure 3B); whether Diego is present on one or both sides of the boundary is impossible to resolve by light microscopy. The localization persisted during hair formation (which occurred at ~30 hr in our stocks) (Figure 3C). These experiments show that Diego becomes localized to the proximal-distal junctional region when the Frizzled signaling pathway is actively generating planar polarity.

To ask whether Frizzled activity was required for polarized Diego localiza-



D

allele	omatidia w/ polarity defects (%)	symmetrical omatidia (%)	n
dgo-269	10.4+/-1.6	6.4+/-2.6	656
dgo-195	15.5+/-6.2	7.2+/-5.1	493
dgo-308	25.5+/-9.2	16.9+/-8.8	650
dgo-380	38.4+/-11.2	28.6+/-13.1	1474

Figure 2. Homozygous *dgo* Flies Have Altered Tissue Polarity in the Wing and Eye

(A) Wing from a homozygous dgo^{380} fly. The region between the fourth and fifth wing vein is shown.

(B) Tangential section through the equatorial region of a homozygous dgo 380 eye.

(C) Schematic presentation of polarity defects shown in (B). The blue line indicates the equator. Black bent arrows represent ommatidia of correct chirality and orientation. Red bent arrows represent misrotated ommatidia. Green arrows are highlighting ommatidia with the wrong chirality (when bent) or symmetrical, achiral ommatidia (when straight).

(D) Allelic quantification of *dgo* eye phenotypes. Despite the overall similarities to *fz* and *dsh* mutant eyes, *dgo* mutant eyes show two distinct features. First, they still retain an appreciable mirror-image symmetry line (equator), and second, there are more achiral, symmetrical ommatidia in *dgo* mutants as compared with *fz* or *dsh* null eyes, suggesting that *dgo* affects a slightly different aspect of polarization. In addition to the polarity eye phenotype, *dgo* mutant eyes also display some defects in cellular differentiation. In particular, the light-harvesting organelles, the rhabdomeres, appear to be partly malformed and the integrity of some cell adhesive properties might be disturbed appearing as "holes" in the microscopic sections.

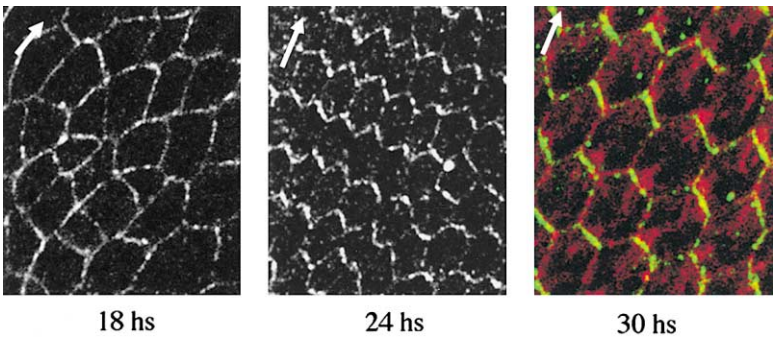


Figure 3. Polarization of Diego during Pupal Wing Development

(A) Diego is uniformly distributed around cell boundaries at 18 hr after puparium formation.

(B) Diego is localized to proximal-distal boundaried by 24 hr after puparium formation.

(C) Diego (green) remains localized as hair formation initiates (filamentous actin in wing hairs is stained red by rhodamine phalloidin).

tion in clones of *frizzled* null tissue. We found that Diego protein was undetectable at the cortex between *frizzled* mutant cells (Figures 4A–4C). In contrast, Diego accumulates to a higher level at boundaries between wild-type and *frizzled* mutant cells, although it is not possible to resolve whether Diego is present on both sides of the boundary. These data suggest that Diego accumulates at boundaries between cells with different levels of Frizzled signaling activity.

Frizzled mutant clones nonautonomously reorganize the polarity of hairs distal to the mutant tissue, and the effect is most pronounced on the medial side of the clone (Adler et al., 2000). Correspondingly, Diego localization in cells on the distal/medial side of *frizzled* clones is reoriented (Figures 6A–6C), suggesting that perturbations in Frizzled signaling can nonautonomously repolarize the distribution of Diego.

To confirm that Diego localizes to boundaries between cells with different levels of Frizzled signaling activity, we examined its distribution in wings expressing Frizzled under the control of *ptc*:GAL4. This produces levels of Frizzled protein that change with distance from the AP boundary. In these wings, Diego relocalized to reflect the artificial gradient of Frizzled expression generated by *Ptc:GAL4* (Figures 4D–4F). In the cells posterior to Frizzled-overexpressing cells, Diego was also relocalized to reflect the nonautonomous disruption of polarity caused by Frizzled. Taken

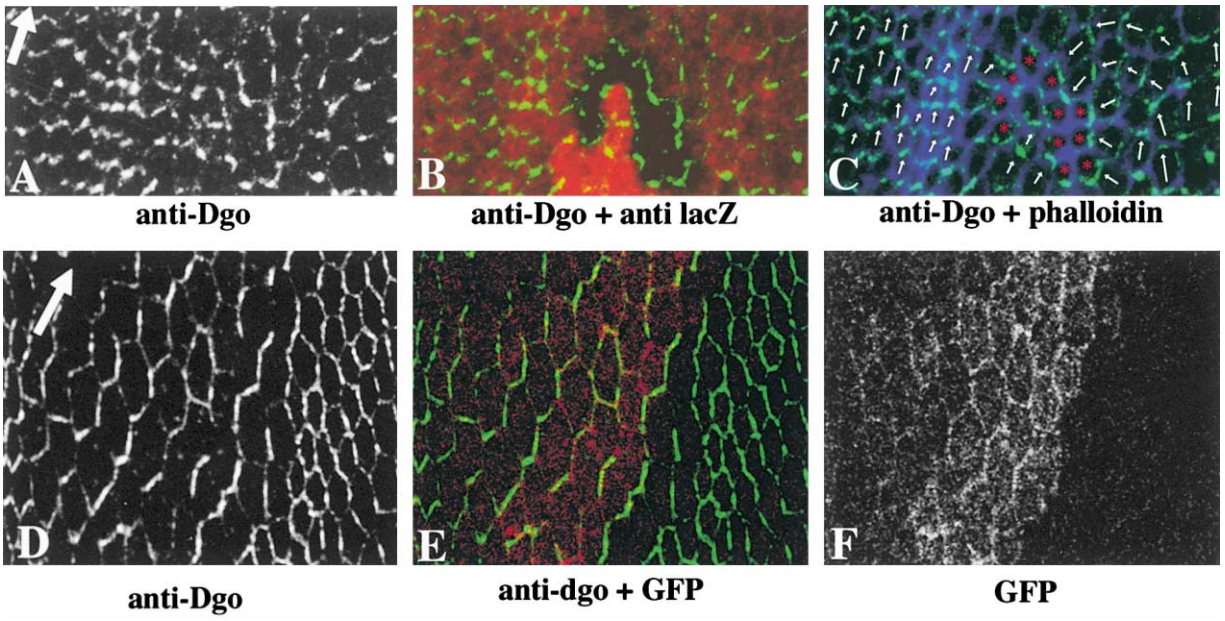


Figure 4. Diego Localizes in Response to Differences in Frizzled Activity

The white arrow points distally. In (A), (B), and (C), the medial region of the wing is to the right.

(A–C) Diego distribution in and around a *frizzled* mutant clone. (A) Diego alone (B) Diego (green) and lacZ (red). The absence of LacZ staining marks *frizzled* mutant cells. The twin spot, which contains two copies of lacZ (and two copies of *frizzled*), is more brightly stained than the wild-type cells that express only one copy. Diego (green) is missing from cell boundaries within the *frizzled* clone and accumulates at boundaries between mutant and wild-type cells. (C) Diego is green, and cell boundaries are labeled by phalloidin (blue). The *frizzled* mutant cells are indicated by red asterisks. Arrows within cells outside the clone indicate their polarity, inferred from Diego localization. The cells on the distal/medial side of the clone have altered polarity.

(D–F) Diego relocalizes in response to *frizzled* overexpression; *frizzled*-overexpressing cells are labeled by the coexpressed marker GFP-GPI (red). Diego (green) is relocalized perpendicular to the normal proximal-distal axis (indicated by the arrow). The cells with smaller apical cross-section near the Frizzled overexpression domain correspond to vein 4.

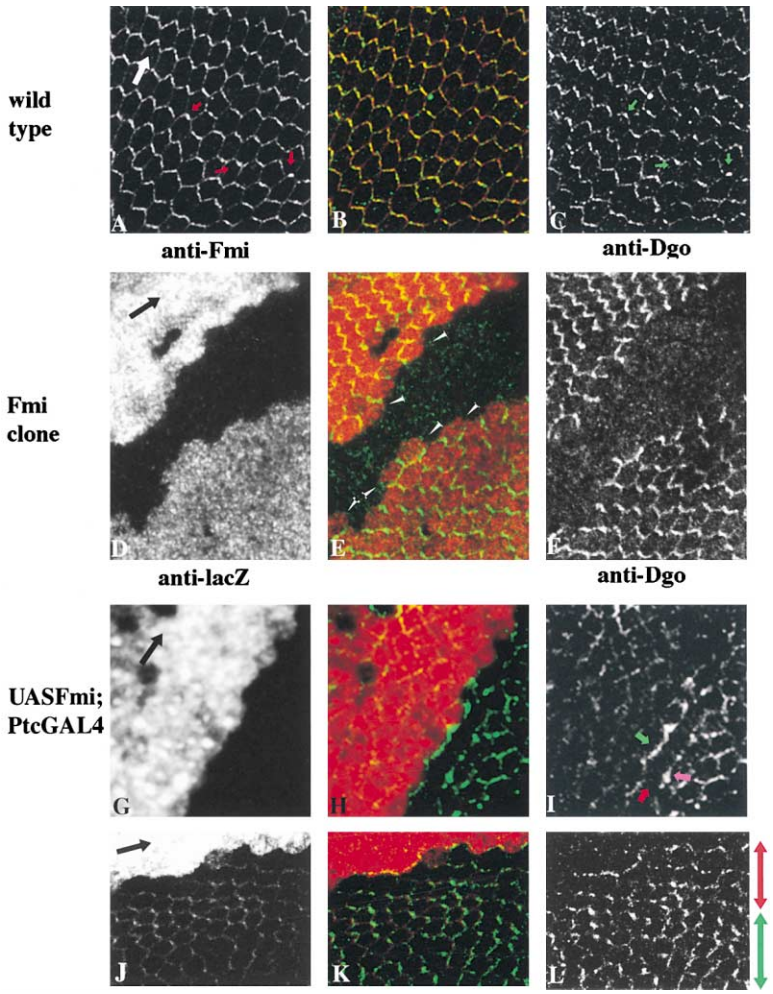


Figure 5. Flamingo Colocalizes with Diego and Is Required for Its Accumulation at Cell Boundaries

Black and while arrows point distally.

(A–C) Colocalization of Flamingo (A and B) and Diego (B and C). Flamingo is shown in red and Diego in green. The arrow indicates the proximal-distal axis. Red and green arrows indicate some of the spots where both Diego and Flamingo are concentrated.

(D–F) Diego is missing from cell boundaries of *flamingo* mutant cells and from interfaces between mutant and wild-type cells.

(G–I) Diego is depolarized by *flamingo* overexpression. (G and H) Flamingo (red) accumulates both at cell boundaries (arrowhead) and on cell-internal structures when overexpressed. (H and I) Diego (green) is no longer localized to proximal distal boundaries in *flamingo*-overexpressing cells. Diego in the first row of wild-type cells abutting the overexpressers accumulates at the overexpression interface (green arrow) and is depleted from proximal-distal boundaries (red arrow). Diego is also elevated on the side of the cell opposite to the overexpression interface (purple arrow).

(J–L) Flamingo (red) and Diego (green) reorient their polarity in response to Flamingo overexpression. The green arrow indicates regions of normal polarity, and the red arrow indicates the region of altered polarity near the overexpression stripe.

together, these data show that apposition of cells with different levels of Frizzled causes the accumulation of Diego at the boundary between them. They further show that the nonautonomous disruption of polarity produced by altering Frizzled signaling results in the mispolarization of Diego.

Diego Colocalizes with Flamingo

The localization of Diego to proximal-distal boundaries resembles that of Flamingo, a homophilic adhesion molecule with seven-transmembrane domains (Usui et al., 1999). To investigate the extent of colocalization between Diego and Flamingo, we stained pupal wings with antibodies to both proteins. Neither protein is smoothly localized at proximal-distal boundaries; instead, the proteins are more abundant at specific spots along the membrane (Figures 5A–5C). Diego and Flamingo show a strong tendency to be especially concentrated at the same places (arrowheads). This suggests that the two proteins are present in the same specialized regions of the membrane.

Flamingo Is Necessary for Cortical Accumulation of Diego

To ask whether cortical localization of Diego depended on Flamingo, we examined the distribution of Diego in clones of Flamingo mutant cells (Figures 5D–5F). Diego was undetectable at the cortex of *flamingo* mutant cells and was also absent from both proximal and distal interfaces between wild-type and *flamingo* mutant tissue. These data suggest that Diego is localized to the cortex via Flamingo, either directly or indirectly. Like Diego, Flamingo also fails to accumulate at either the proximal or distal interface between wild-type and Flamingo mutant clones (Usui et al., 1999). This has been interpreted to mean that Flamingo is normally enriched both proximally and distally and that homophilic interactions are required for its polarized accumulation. Similarly, we would argue that Diego is normally enriched on both proximal and distal sides of the cell and that stable cortical Diego localization occurs only where homophilic Flamingo interactions are possible.

Flamingo Is Not Sufficient for Diego Localization

Clearly, Flamingo is required for cortical accumulation of Diego. To ask whether it was also sufficient, that is, whether Diego simply accumulates wherever Flamingo is present, we examined the distribution of Diego in cells that overexpressed Flamingo (Figures 5G–5I). Although some Flamingo can be detected at cell boundaries when the protein is overexpressed, Flamingo accumulates most obviously in bright spots inside the cell. Since

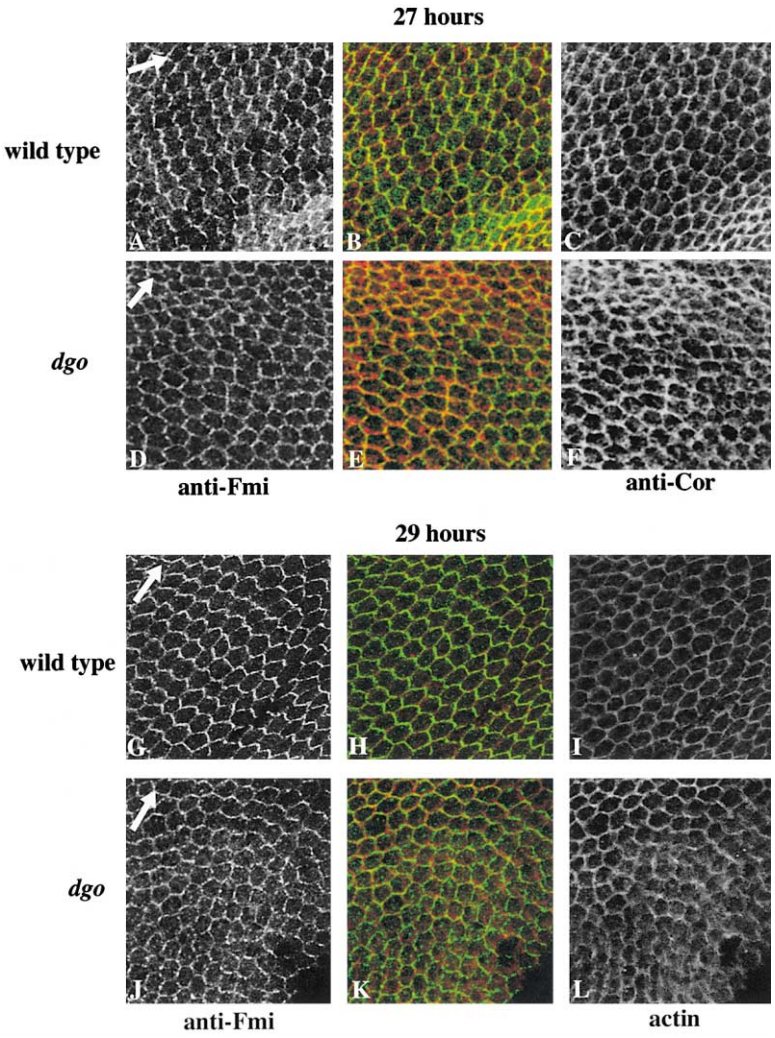


Figure 6. Flamingo Accumulation Is Controlled by Diego

White arrows point distally.

(A–C) Polarized localization of Flamingo in wild-type pupal wings 27 hr after puparium formation.

(D–F) A 27 hr *diego* mutant wing stained in parallel to that shown in (A–C) and imaged under identical conditions. (D and E) Flamingo (green) is depolarized (E and F). Coracle (red) is normally distributed.

(G–I) A wild-type pupal wing 29 hr after puparium formation imaged between veins 4 and 5. (G and H) Flamingo (green). (H and I) actin (red).

(J–L) A *dgo380* 29 hr pupal wing imaged under identical conditions in the same region. (J and K) Flamingo (green) is abnormally polarized in whorls. (L) Actin (red) organization is normal.

Flamingo is a transmembrane protein, these spots might either represent protein that is trapped in the secretory pathway or that has accumulated in the endocytic pathway. In Flamingo-overexpressing cells, Diego protein appears less abundant on the cortex overall, and what Diego remains is no longer restricted to proximal-distal cell boundaries. Diego does not colocalize with Flamingo inside the cell where Flamingo accumulates to the highest level. This indicates that other proteins besides Flamingo must promote the cortical localization of Diego. We also failed to observe colocalization of Diego and Flamingo upon transfection of S2 cells (data not shown), indicating that other proteins may regulate whether Diego and Flamingo are present in the same complexes. Nevertheless, at least at the cortex, dominant mislocalization of Flamingo by overexpression can relocalize Diego.

Overexpression of Flamingo Nonautonomously Reorients Both Diego and Flamingo Polarization

In wild-type cells adjacent to Flamingo-overexpressing cells, Diego disappears from the proximal-distal boundaries (red arrow in Figure 5I) and appears to accumulate to a higher level at the boundary with the Flamingo-overexpression domain, perpendicular to its normal proximal-distal pattern (green arrow in Figure 5I). Furthermore, overaccumulation of Diego is also seen on the cell boundary opposite to that which is contacting the Flamingo-overexpressing cells (purple arrow in Figure 5I). In some wings, reorientation of both Diego and Flamingo occurs up to three cell diameters away from the overexpression stripe (Figure 5J–5L). Thus, despite the fact that loss of Flamingo activity in clones has an essentially autonomous effect, depleting Diego and Flamingo only from the directly adjacent cell boundary, *flamingo* overexpression causes nonautonomous relocalization of both itself and Diego over several cell diameters.

Diego Promotes the Proximal-Distal Polarization of Flamingo

To ask whether Diego was required for Flamingo localization, we compared the distribution of Flamingo in wild-type versus homozygous *diego* mutant wings. In all of the wild-type wings we examined, the Flamingo protein was enriched on proximal-distal boundaries by 27 hr after puparium formation (Figures 6A and 6B). In contrast, Flamingo in *dgo380* wings localizes uniformly around the junctional region at this time (Figures 6D and

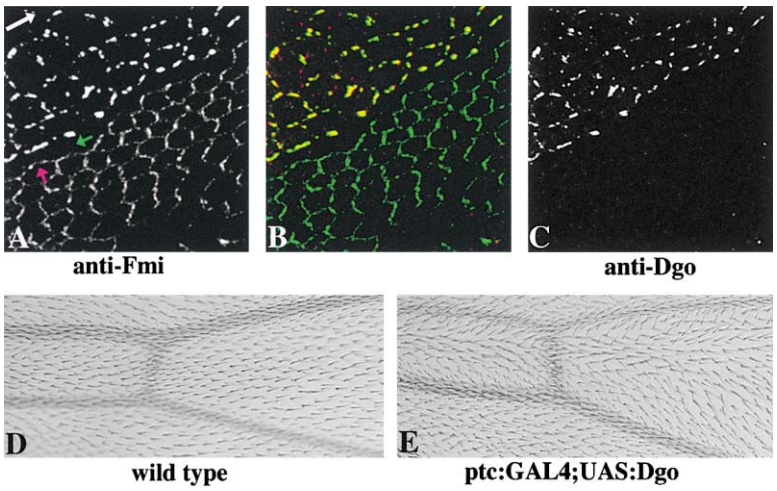


Figure 7. Flamingo Coaccumulates with Overexpressed Diego

(A and B) Flamingo (green) accumulates to abnormally high levels in junctional clusters in Diego-overexpressing cells. The white arrow points distally. The green arrow indicates depletion from a proximal distal boundary in a cell next to Diego overexpressers. The purple arrow lies in a cell in which Flamingo is no longer polarized along the proximal distal and indicates higher than normal levels of Flamingo on the posterior (as opposed to proximal/distal) boundary.

(B and C) Overexpressed Diego (red) colocalizes with Flamingo.

(D and E) Repolarization of wing hairs in cells overexpressing Diego. (D) Hairs point distally in a wild-type wing. (E) Diego overexpressed under the control of the Patched promoter reorients wing hairs away from the high point of expression.

6E), similar to its distribution in *frizzled* mutant wings (Usui et al., 1999). We quantified the ratio of Flamingo staining intensity on proximal-distal versus anterior-posterior cell boundaries in images from four wild-type wings and four *dgo380* mutant wings. In wild-type wings, Flamingo was enriched between 2.5- and 3.2-fold on proximal-distal boundaries at 27 hr after puparium formation. In contrast, the Flamingo in *dgo380* mutant wings was not significantly enriched (ratios ranged between 0.8 and 1.3). These data suggest that Diego promotes Flamingo polarization.

Flamingo sometimes polarizes its distribution in parts of *dgo380* mutant wings at later times (29–30 hr after puparium formation), but the axis of polarity is often abnormal, presaging the whorls of misoriented hairs observed in *dgo380* adult wings (compare Figures 6G and 6H with Figures 6J and 6K). In all cases, the effect of *diego* is specific to Flamingo and does not affect the distribution of cortical actin (Figure 6L) or Coracle (Figure 6F). Clonal analysis showed that *diego* loss of function does not affect Flamingo localization outside of *diego* clones (data not shown). These data suggest that Diego increases the efficiency of Flamingo polarization and promotes its correspondence with the proximal-distal axis determined by Frizzled. The fact that Flamingo is localized similarly in *frizzled* and *diego* mutant cells may indicate that Frizzled acts through Diego to mediate Flamingo polarization.

Overexpressed Diego Drives Depolarized Clustering of Flamingo and Reorients Flamingo Distribution in Adjacent Cells

To assess the effects of excess Diego protein on polarity, we examined the effect of Diego overexpression on Flamingo localization. When Diego protein is overexpressed with the *ptc:GAL4* driver, it still localizes exclusively to the junctional region, but is present in large aggregates with no proximal-distal polarity (Figure 7C). Flamingo protein accumulates in these cortical clusters, and its distribution is no longer polarized along the proximal-distal axis (Figures 7A and 7B). The effect of Diego overexpression on Flamingo localization is specific, as neither Cadherin nor Coracle coaccumulates with Diego (data not shown). These data suggest that Diego specifically promotes clustering of Flamingo. We suggest that under wild-type conditions, Diego-mediated Flamingo clustering is regulated in response to Frizzled signaling and occurs only at proximal-distal boundaries.

Interestingly, Diego overexpression has nonautonomous effects on Flamingo localization in adjacent cells. In the first row of wild-type cells adjacent to Diego overexpressers, Flamingo is missing from proximal-distal boundaries (green arrow) and presumably relocalizes to maximize homophilic interactions with the clustered Flamingo on the Diego-overexpressing cells (red arrow). Interestingly, Flamingo protein levels are also modestly elevated on the cell boundary opposite to the Diego-overexpressing cells (purple arrow). As a result, the Flamingo in the second row of cells seems less unambiguously polarized to proximal-distal boundaries than in the cells at a greater distance from the overexpression stripe.

Diego and Flamingo Act Antagonistically to Orient the Proximal-Distal Axis

The ability of adjacent pupal wing cells to compare the activity of different tissue polarity genes and orient hairs accordingly is intrinsic to the mechanism of planar polarization. When artificial expression gradients are produced, Frizzled and Flamingo cause opposite effects on hair polarity; hairs point away from cells with higher Frizzled expression but toward cells with higher Flamingo expression. To ask how cells with differing levels of Diego behaved, we examined the adult wings from UAS:*dgo* * *ptc:*GAL4 crosses. We found that wing hairs were oriented from high- to low- expressing cells (Figure 7E) in *ptc:*GAL4;UAS:*dgo* wings—similar to the effects caused by Frizzled and opposite to those caused by Flamingo (Usui et al., 1999). These data suggest that Diego responds positively to Frizzled signaling and that the subsequent Diego-mediated clustering of Flamingo has negative effects on its signaling activity.

Discussion

The Frizzled family of seven-pass transmembrane proteins mediates cell polarization events in many different

tissues and organisms (Rocheleau et al., 1997; Gho and Schweisguth, 1998; Lu et al., 1998; Shulman et al., 1998; Djiane et al., 2000, Heisenberg et al., 2000; Wallingford et al., 2000), but the mechanism by which they do so is not well understood. In wing epithelial cells, one consequence of Frizzled signaling is the polarization of the seven-pass transmembrane Cadherin Flamingo, a homophilic adhesion molecule, to proximal and distal cell boundaries. How Frizzled signaling causes polarization of Flamingo along the planar axis is not clear. We have identified a gene, *diego,* whose protein product is required for normal polarization of Flamingo. Diego is itself localized at proximal-distal boundaries in response to Frizzled signaling. It comprises six ankyrin repeats at its N terminus; because ankyrin repeats can mediate protein-protein interactions, Diego has the potential to act as a scaffold for the assembly of specific multiprotein complexes. Diego is homologous to a human cDNA of unknown function, suggesting that Diego-like proteins may work in similar pathways in other species.

In the wing, proximal-distal localization of Diego and Flamingo proteins is interdependent. Perturbing the distribution of either protein disrupts that of the other. In the absence of Flamingo, Diego fails to localize to the cortex at all. On the other hand, in *diego* mutant cells, Flamingo is cortically localized but fails to polarize efficiently or accurately along the proximal-distal axis. The localization of each of these proteins can also be perturbed by overexpression of the other. When Diego is overexpressed, it accumulates in large nonpolarized junctional clusters that recruit high levels of Flamingo protein as well. In contrast, overexpression of Flamingo has a dispersive rather than a clustering effect on Diego in the same cell, causing it to delocalize from proximal-distal boundaries and assume a more uniform cortical distribution. Furthermore, in cells adjacent to Flamingo overexpressers, Diego is dominantly relocalized with Flamingo to face the overexpression domain. Clearly, Flamingo and Diego each affect the localization of the other.

One model that would explain these data is that Diego and Flamingo interact either directly or indirectly within the same multiprotein complexes. We have been unable to address the possibility of a direct interaction between Diego and Flamingo by coimmunoprecipitation because Diego remains insoluble in detergent under conditions that solubilize Flamingo (B. Hartmann and S. Eaton, unpublished). Diego might associate with the membrane via such complexes and subsequently help to polarize their localization in response to Frizzled signaling. We feel that Diego's cortical localization and ability to cluster make this model more likely than one in which Diego promotes polarized delivery of the Flamingo protein from the Golgi apparatus.

How might Diego and Flamingo polarize in response to Frizzled? Although one possibility is that Diego mediates polarization by directly interacting with components of the activated Frizzled pathway, the interdependence of Flamingo and Diego localization speaks against such a linear model. A more reasonable model is that localized Frizzled signaling might modify the properties of the Diego protein at proximal-distal boundaries, increasing its ability to promote clustering of itself and Flamingo. This initial bias in localization caused by clustering might then be potentiated via homophilic interactions between Flamingo molecules on adjacent cells. This second model is more consistent with the reciprocal effects that Flamingo and Diego have on each other's localization.

The ability of polarization signals to propagate from cell to cell is one of the most intriguing aspects of tissue polarity and is one of the least understood. It has been known for some time that locally perturbing Frizzled signaling has long-range effects on the polarity of the surrounding tissue (Vinson and Adler, 1987). This repolarization is reflected, at the molecular level, by the altered distribution of Flamingo and Diego (discussed in this article; Usui et al., 1999). Interestingly, we see that, in the absence of any direct disruption of Frizzled, Flamingo overexpression causes nonautonomous repolarization of Diego and Flamingo that propagates over several cell diameters. Both Flamingo and Diego accumulate ectopically to a high level at the interface between *frizzled* mutant and wild-type tissue (discussed in this article; Usui et al. 1999). Our data suggest that the accumulation of Diego and Flamingo at *frizzled* clonal boundaries might play an important role in propagating repolarization of these proteins into the surrounding tissue.

How might this propagation occur? Although it is easy to understand how Flamingo-mediated homophilic interactions could recruit Flamingo and Diego from a directly adjacent cell to the boundary with the overexpression domain, it is less clear how this would result in a corresponding accumulation on the opposite side of the cell. One possibility is that when Diego and Flamingo are polarized via Flamingo-mediated homophilic interactions, a locally acting intracellular signal is generated that prevents similar interactions nearby. Flamingo and Diego might then tend to accumulate on the opposite side of the cell, where such interactions were not discouraged. Increased levels of Diego and Flamingo on the opposite side of the cell might, in turn, recruit Diego and Flamingo from the next cell in line. Such a mechanism might be sufficient to propagate polarization of these proteins over many cell diameters.

Experimental Procedures

Molecular Cloning and Sequencing

The genomic DNA surrounding EP(2)2619 was cloned by plasmid rescue using standard techniques, and both it and the homologous EST were sequenced by M.W.G. (Munich, Germany).

Generation of *dgo* Mutations

Mutations in *diego* (corresponding to CG12342 in the Gadfly database) were induced by imprecise excision of EP(2)2619 and screened over Df(2R)27. Those excisions that produced phenotypes or lethality over the deficiency were mapped using polymerase chain reaction (PCR). EP(2)2619 is inserted upstream of *diego* and downstream of C12323, which encodes a putative proteosome subunit. Forward primers located upstream of EP(2)2619, but downstream of CG12323, were used in combination with reverse primers located either within the coding region of *diego* or downstream of the *diego*-transcribed region. We found that all three excisions that impinged on CG12323 were lethal, whereas those that were unidirectional and terminated within the *diego* transcript were homozygous viable with tissue polarity defects. Those unidirectional excisions that proceeded beyond the *diego* transcript were also lethal. Genetic analysis of these lethal excisions identified one, ex(212), with an end point downstream of *diego* and upstream of *flamingo* (which is located ~18 kb downstream of *diego*). We used this small deficiency to

determine whether the homozygous viable *diego* mutations produced stronger wing phenotypes when hemizygous. The fact that they did not indicated that the viable *diego* alleles were at least strong hypomorphs in the wing.

Antibodies

A GST-Diego fusion protein comprising 25 kb C-terminal to the ankyrin repeats was used to immunize rabbits. Antisera were affinity purified and used at dilutions between 1:100 and 1:250. Other antibodies used were as follows: mouse anti-Flamingo (Usui et al., 1999) and guinea pig anti-Coracle (Fehon et al., 1994). Rhodamine-conjugate phalloidin was obtained from Molecular Probes Inc. (Eugene, OR). Pupal wings were processed for immunocytochemistry as previously described (Eaton et al., 1995) and were imaged with a Zeiss LSM510 confocal microscope.

Quantification of Flamingo Staining

Using the plot profile function of NIH image, staining intensity was measured along single lines running through at least four cell boundaries, along either the pd or ap axis. A total of at least 16 cell boundaries of each type was quantified per image. In four separate wild-type wings, we found pd:ap ratios of 2.7:1, 2.5:1, 3.0:1, and 3.2:1. In four separate Dgo mutant wings, the ratios were 1.3:1, 1.1:1, 0.8:1, and 1.1:1.

Histology

Sectioning and microscopic analysis of adult eyes were performed as previously described (Tomlinson and Ready, 1987).

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References

Adler, P., Charlton, J., Jones, K., and Liu, J. (1994). The cold-sensitive period for *frizzled* in the development of wing hair polarity ends prior to the start of hair morphogenesis. Mech. Dev. *46*, 101–107.

Adler, P.N., Krasnow, R.E., and Liu, J. (1997). Tissue polarity points from cells that have higher Frizzled levels towards cells that have lower Frizzled levels. Curr. Biol. *7*, 940–949.

Adler, P., Taylor, J., and Charlton, J. (2000). The domineering nonautonomy of frizzled and van gogh clones in the Drosophila wing is a consequence of a disruption in local signalling. Mech. Dev. *96*, 197–207.

Chae, J., Kim, M.J., Goo, J.H., Collier, S., Gubb, D., Charlton, J., Adler, P.N., and Park, W.J. (1999). The Drosophila tissue polarity gene starry night encodes a member of the protocadherin family. Development *126*, 5421–5429.

Djiane, A., Riou, J., Umbhauer, M., Boucaut, J., and Shi, D. (2000). Role of frizzled 7 in the regulation of convergent extension movements during gastrulation in Xenopus laevis. Development *127*, 3091–3100.

Eaton, S. (1997). Planar polarization of Drosophila and vertebrate epithelia. Curr. Opin. Cell Biol. *9*, 860–866.

Eaton, S., Auvinen, P., Luo, L., Jan, Y.N., and Simons, K. (1995). CDC42 and Rac1 control different actin-dependent processes in the *Drosophila* wing disc epithelium. J. Cell Biol. *131*, 151–164.

Eaton, S., Wepf, R., and Simons, K. (1996). Roles for Rac1 and Cdc42 in planar polarization and hair outgrowth in the wing of *Drosophila*. J. Cell Biol. *135*, 1277–1289.

Fehon, R., Dawson, I., and Artavnis-Tsakonas, S. (1994). A *Drosophla* homologue of membrane-skeleton protein 4.1 is associated with septate junctions and is encoded by the *coracle* gene. Development *120*, 545–557.

Gho, M., and Schweisguth, F. (1998). Frizzled signalling controls orientation of asymmetric sense organ precursor cell divisions in Drosophila. Nature *393*, 178–181.

Gubb, D., Green, C., Huen, D., Coulson, D., Johnson, G., Tree, D., Collier, S., and Roote, J. (1999). The balance between isoforms of the prickle LIM domain protein is critical for planar polarity in Drosophila imaginal discs. Genes Dev. *13*, 2315–2327.

Heisenberg, C.P., Tada, M., Rauch, G.J., Saude, L., Concha, M.L., Geisler, R., Stemple, D.L., Smith, J.C., and Wilson, S.W. (2000). Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. Nature *405*, 76–81.

Krasnow, R.E., Wong, L.L., and Adler, P.N. (1995). Dishevelled is a component of the frizzled signaling pathway in Drosophila. Development *121*, 4095–4102.

Lu, B., Jan, L.Y., and Jan, Y.N. (1998). Asymmetric cell division: lessons from flies and worms. Curr. Opin. Genet. Dev. *8*, 392–399.

Mlodzik, M. (1999). Planar polarity in the Drosophila eye: a multifaceted view of signaling specificity and cross-talk. EMBO J. *18*, 6873–6879.

Reifegerste, R., and Moses, K. (1999). Genetics of epithelial polarity and pattern in the Drosophila retina. Bioessays *21*, 275–285.

Rocheleau, C.E., Downs, W.D., Lin, R., Wittmann, C., Bei, Y., Cha, Y.H., Ali, M., Priess, J.R., and Mello, C.C. (1997). Wnt signaling and an APC-related gene specify endoderm in early C. elegans embryos. Cell *90*, 707–716.

Rorth, P. (1996). A modular misexpression screen in Drosophila detecting tissue-specific phenotypes. Proc. Natl. Acad. Sci. USA *93*, 12418–12422.

Shulman, J.M., Perrimon, N., and Axelrod, J.D. (1998). Frizzled signaling and the developmental control of cell polarity. Trends Genet. *14*, 452–458.

Taylor, J., Abramova, N., Charlton, J., and Adler, P.N. (1998). Van Gogh: a new Drosophila tissue polarity gene. Genetics *150*, 199–210.

Tomlinson, A., and Ready, D.F. (1987). Cell fate in the Drosophila Ommatidium. Dev. Biol. *123*, 264–272.

Tomlinson, A., Strapps, W.R., and Heemskerk, J. (1997). Linking Frizzled and Wnt signaling in Drosophila development. Development *124*, 4515–4521.

Usui, T., Shima, Y., Shimada, Y., Hirano, S., Burgess, R.W., Schwarz, T.L., Takeichi, M., and Uemura, T. (1999). Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. Cell *98*, 585–595.

Vinson, C., and Adler, P.N. (1987). Directional non-cell autonomy and the transmission of polarity information by the *frizzled* gene of *Drosophila*. Nature *329*, 549–551.

Wallingford, J.B., Rowning, B.A., Vogeli, K.M., Rothbacher, U., Fraser, S.E., and Harland, R.M. (2000). Disheveled controls cell polarity during Xenopus gastrulation. Nature *405*, 81–85.

Wolff, T., and Rubin, G.M. (1998). Strabismus, a novel gene that regulates tissue polarity and cell fate decisions in Drosophila. Development *125*, 1149–1159.

Wong, L.L., and Adler, P.N. (1993). Tissue polarity genes of Drosophila regulate the subcellular location for prehair initiation in pupal wing cells. J. Cell Biol. *123*, 209–221.

Zheng, L., Zhang, J., and Carthew, R.W. (1995). frizzled regulates mirror-symmetric pattern formation in the Drosophila eye. Development *121*, 3045–3055.