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# *DWnt4* Regulates Cell Movement and Focal Adhesion Kinase during *Drosophila* Ovarian Morphogenesis

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## Summary

**Cell motility is regulated by extracellular cues and by intracellular factors that accumulate at sites of contact between cells and the extracellular matrix. One of these factors, focal adhesion kinase (FAK), regulates the cycle of focal adhesion formation and disassembly that is required for cell movement to occur. Recently, Wnt signaling has also been implicated in the control of cell movement in vertebrates, but the mechanism through which Wnt proteins influence motility is unclear. We demonstrate that *Drosphila* Wnt4 is required for cell movement and FAK regulation during ovarian morphogenesis. Dfrizzled2, Disheveled, and protein kinase C are also required. The DWnt4 cell motility pathway is distinct from both the canonical Wnt pathway and the planar polarity pathway. Our data suggest that DWnt4 facilitates motility through regulation of focal adhesions.**

## Introduction

The regulation of cell motility requires the coordination of extracellular and intracellular signaling factors that impinge on the organization of the cytoskeleton (for reviews, see Critchley [2000], Gumbiner [1996], Lauffenburger and Horwitz [1996], and Parsons et al. [2000]). Many of the intracellular factors are localized to focal complexes or focal adhesions, which serve to anchor the cytoskeleton to the extracellular surface and to provide traction as the plasma membrane extends forward. Cell movement begins with extension of actin projections that are stabilized at the leading edge of the cell by focal complexes. As the cell surface continues to extend forward, these complexes become displaced from the leading edge and are referred to as focal adhesions. Focal adhesions are disassembled at the lagging edge of the cell, allowing the cell to move forward. The cyclical regulation of focal complex formation and disassembly is critical in the control of cell movement, and factors involved in this regulation are often localized to the complexes themselves.

Integrins are key components of these complexes, as they form the bridge between actin and the extracellular matrix. Several cytoplasmic protein tyrosine kinases, including Src and focal adhesion kinase (FAK), are also present in focal complexes (Critchley, 2000; Liu et al., 2000). The localization of these kinases to focal complexes leads to their activation and the subsequent phosphorylation of their targets. FAK phosphorylates focal adhesion components to induce focal adhesion disassembly. It can also function upstream of PI3 kinase, extracellular signal-regulated kinase (ERK), and Jun-N-terminal kinase (JNK) to regulate diverse cellular processes, such as cell proliferation and viability (Parsons et al., 2000).

Recently, the Wnt family of secreted glycoproteins has also been implicated in the regulation of cell movement. Wnt proteins form a large group of secreted ligands that regulate many cellular processes, including cell fate specification, cell proliferation, epithelial/mesenchymal transitions, cell adhesion, and cell movement (McEwen and Peifer, 2000; Patapoutian and Reichardt, 2000; Polakis, 2000). Many of these processes are mediated through a canonical pathway, ultimately resulting in the stabilization and nuclear translocation of β-catenin. In this pathway, Wnt proteins activate the cytoplasmic protein Disheveled (Dsh) through association with members of the Frizzled (Fz) and LDL-Receptor families (Cadigan and Nusse, 1997; Pinson et al., 2000; Wehrli et al., 2000). In the absence of Wnt signaling, β-Catenin is targeted to the proteasome for degradation. However, in the presence of canonical Wnt signaling, β-catenin accumulates and is translocated to the nucleus, where it complexes with TCF family DNA binding proteins and regulates the transcription of target genes. Although this pathway mediates many Wnt-regulated processes, it does not appear to be involved in Wnt regulation of cell movement (Heisenberg et al., 2000; Tada and Smith, 2000; Wallingford et al., 2000).

Wnt signaling is required during vertebrate gastrulation for cell movement, but the cellular and molecular mechanisms underlying Wnt regulation of cell movement are poorly defined. Two aspects of cell movement during gastrulation are regulated by Wnt signaling: the separation of moving cells from the adjacent cell layer and the stabilization of actin projections during convergent extension. Wnt signaling through Fz7- and $Ca^{2+}$-dependent protein kinase C (PKC) is required for cell separation (Kuhl et al., 2000; Winklbauer et al., 2001). Although the effects of reduced Dsh activity were not examined, the loss of Fz7 can be rescued by PKC but not by Dsh. This suggests that PKC signaling and Dsh have separable roles during gastrulation. The downstream effectors of cell separation have not been identified.

During convergence and extension, Wnt signaling through Dsh is required for the polarized stabilization of lamellipodia (Wallingford et al., 2000). During this process, cells extend lamellipodia in all directions, but these projections are preferentially stabilized in what will be the direction of cell movement. In the presence of an

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interfering form of Dsh, cells randomly extend lamellipodia, which fail to stabilize in the direction of movement. The ligand that mediates convergent extension during zebrafish and *Xenopus* gastrulation is Wnt11 (Heisenberg et al., 2000; Tada and Smith, 2000). Wnt11 mutations disrupt axis elongation in zebrafish and *Xenopus* embryos, and these effects can be rescued by Dsh. This indicates that Wnt proteins regulate lamellipodial behavior through Dsh.

The polarization of actin extensions from moving cells during gastrulation is reminiscent of the establishment of planar polarity in *Drosophila*. In the wing epithelium, this process involves the localization of actin bundles to the distal tips of hexagonally packed cells (Adler, 1992). In this tissue, the actin localization prefigures the formation of actin-based hairs rather than lamellipodial stabilization. However, the similarity in polarized actin organization during cell movement and hair formation suggests that the signal transduction pathways involved in these two processes are similar (McEwen and Peifer, 2000). This idea is supported by the observation that mutant forms of Dsh that abolish function in planar polarity but not in canonical signaling also interfere with Dsh activity during convergent extension (Tada and Smith, 2000; Wallingford et al., 2000). In addition, the *Xenopus* homolog of *strabismus* (*stbm*), which functions in the establishment of planar polarity, has recently been shown to influence gastrulation (Park and Moon, 2001).

Although these analyses suggest that the pathways used by Wnt proteins to facilitate cell movement and the planar polarity pathway are similar, it has been difficult to compare the two directly. No ligand has been identified in the *Drosophila* planar polarity pathway, and Wnt proteins have not been associated with cell movement in *Drosophila*. Mutations in only two of the seven *Drosophila* Wnt genes, Wingless and DWnt2 (Kozopas et al., 1998), have been reported. Wingless (the ortholog of vertebrate Wnt1) signals through the canonical Wnt signaling pathway (Cadigan and Nusse, 1997). The signal transduction mechanism used by DWnt2 has not been characterized, but it appears to engage the canonical pathway in certain tissues (Llimargas and Lawrence, 2001).

We have generated mutations in a third *Drosophila* Wnt gene, DWnt4 (Graba et al., 1995), and show that it facilitates cell movement during ovarian organogenesis through a signaling mechanism that results in FAK accumulation. Our data indicate that a canonical pathway receptor, DFz2 (Bhanot et al., 1999), is the primary receptor for DWnt4 in facilitating cell movement. However, the downstream effector of canonical signaling, TCF (van de Wetering et al., 1997), is not required for DWnt4-mediated cell movement. A mutation in Dsh that specifically disrupts planar polarity (Boutros and Mlodzik, 1999) disrupts cell movement, but other planar polarity mutants do not exhibit a *DWnt4*-like phenotype. Finally, we find that inhibition of PKC impedes cell movement. We present a model in which DWnt4 promotes motility and regulates FAK through a distinct mechanism that brings together components from multiple Wnt pathways.

## Results

DWnt4 is most closely related to vertebrate Wnts 9, 14, and 15 (Bergstein et al., 1997; Graba et al., 1995). The primary feature that distinguishes these proteins from other Wnt family members is an unusual spacing of conserved cysteine residues at the C terminus of each protein. This feature, and the observation that DWnt4 can elicit a response that is distinct from that of Wingless in the embryonic epidermis, led us to postulate that DWnt4 signals through a noncanonical signaling mechanism (Buratovich et al., 2000; Gieseler et al., 1999; Graba et al., 1995).

To analyze *DWnt4* signaling genetically, we generated mutations at the locus (see Experimental Procedures). We isolated three mutant alleles, all of which display partial lethality. The cause of lethality has not been determined; embryos hatch with no obvious defects, but the larvae die in the first or second instar. Approximately 10%–15% of the mutant individuals survive to adulthood. These adults are male and female sterile but exhibit no external defects. Sequence analysis of genomic DNA from the mutants reveals that two of the alleles have changes in the *DWnt4* coding region: a stop signal is generated at position 343 in $DWnt4^{EMS23}$, and a 3 bp deletion removes a highly conserved glutamate residue at position 299 in $DWnt4^{C1}$. The full-length protein is 539 amino acids. For each of these alleles, the lethality and mutant phenotype of *DWnt4*/*DWnt4* is similar to that of *DWnt4*/*Df*, indicating that each allele is either amorphic or strongly hypomorphic. The third allele, $DWnt4^{X1}$, does not contain a mutation in the coding region, suggesting that this allele contains a regulatory mutation. Here we have focused on the phenotype in the developing ovary.

### Ovariolar Structure Is Disrupted in *DWnt4* Mutants

The *Drosophila* adult ovary contains between 13 and 16 chains of developing egg chambers called ovarioles, each of which is contained within an ovariolar sheath (King, 1970) (Figures 1A and 1D). The sheath that covers each ovariole is composed of a layer of squamous epithelium surrounded by bands of muscle (Figure 1D). The sheath epithelium secretes a thick basement membrane and provides structural support to the ovariole.

A specialized group of cells at the apical tip of the germarium, known as the terminal filament cells, are contiguous with the sheath epithelium. Immediately basal to the terminal filaments are germline and somatic stem cells. The germline stem cells divide to produce the oocyte and supporting nurse cells; the somatic stem cells give rise to a layer of follicle cells that surround the germ cells (Spradling, 1993). When the oocyte and nurse cells become enveloped by the follicle cells, they pinch off from the germarium to form an egg chamber, and oogenesis proceeds. This process is repeated so that each ovariole contains a chain of egg chambers with the germarium and young egg chambers at the apical end and mature oocytes at the basal end.

The structure of the ovarioles is disrupted in *DWnt4* mutants (Figure 1C). Wild-type ovarioles exhibit a linear arrangement, with their germaria meeting at the apical tip of the ovary and mature oocytes at the basal end (Figures 1A and 1B). In *DWnt4* mutants, the germaria and younger egg chambers "flop" down beside the older cysts, frequently becoming positioned adjacent to the dorsal appendages of mature oocytes (Figure 1C). We postulated that the disorganization of the mutant ovarioles results from a lack of structural support from the

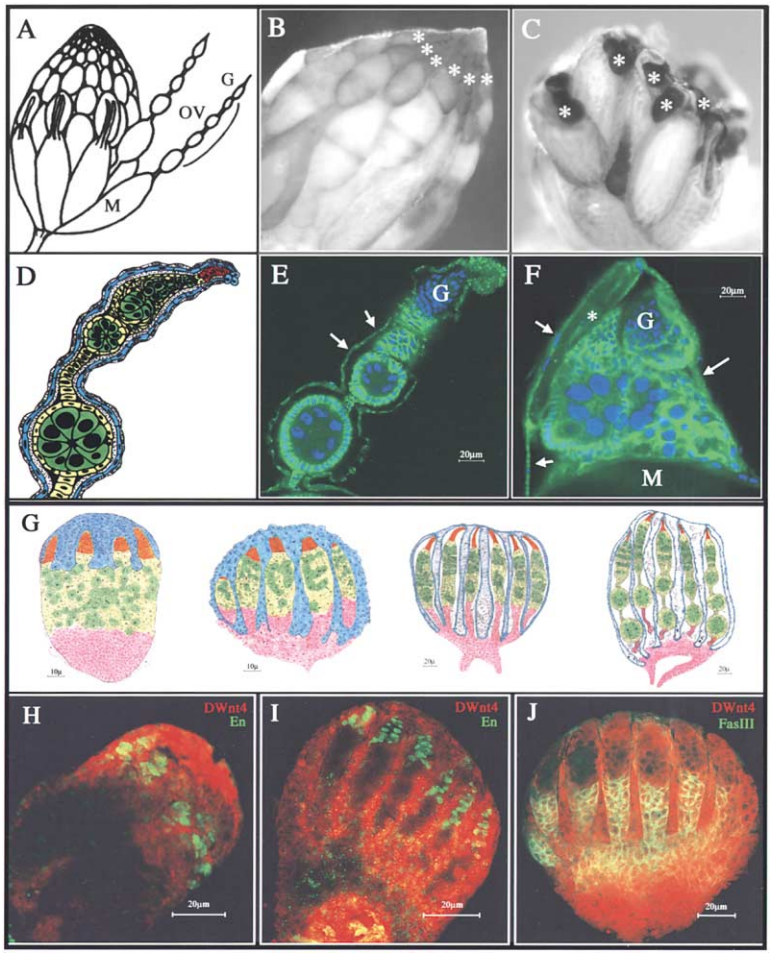


Figure 1. *DWnt4* Mutations Disrupt Ovariolar Structure, and *DWnt4* Is Expressed in the Epithelial Sheath during Morphogenesis

(A) A diagram of the adult *Drosophila* ovary modified from King (King, 1970). Each ovary contains between 13 and 16 ovarioles (Ov) connected to a common oviduct. Each ovariole is a linear string of developing egg chambers that begins with a germarium (G) and ends with mature oocytes (M).

(B and C) Ovaries stained with hematoxylin and eosin. The germaria and young egg chambers are indicated by asterisks.

(B) Wild-type

(C) *DWnt4* mutant.

(D) A drawing of a single ovariole. The ovariolar sheath, which is composed of an inner epithelial layer (blue) and an outer muscular layer (white), supports the ovariole. Terminal filaments, red; germ cells, green; follicle cells, yellow.

(E and F) Ovarioles stained for Fasciclin III (green) and DNA (blue). The sheath (arrows) surrounds the ovariole.

(E) Wild-type.

(F) *DWnt4* mutant.

(G) A cartoon illustrating the development of the *Drosophila* ovary, modified from King (King, 1970).

(H) A wild-type ovary isolated 2 hr APF and stained with antibodies recognizing DWnt4 (red in panels [H], [I], and [J]) and Engrailed, which labels the terminal filament cells (green in panels [H] and [I]); apical is up and to the right.

(I) A similarly stained ovary isolated 16 hr APF.

(J) An ovary isolated 18 hr APF and stained with antibodies recognizing DWnt4 and Fasciclin III, which is expressed by basal stalk cells at this stage (green). We also observe DWnt4 expression in the adult (data not shown), which is restricted to the sheath, the basal half of the terminal filament cells, and regions I and II of the germarium.

ovariolar sheath. We examined the sheath epithelium using an antibody against Fasciclin III, which marks the sheath epithelium and the follicle cells in the adult ovary. In wild-type, the sheath surrounds the ovariole as a glove surrounds a finger (Figure 1E). In *DWnt4* mutant females, the sheath is present, but the ovariolar "finger" is folded, as if the "glove" is too small (Figure 1F). When the sheath is manually removed, however, the ovarioles straighten out and are grossly normal (data not shown). This suggests that the aberrant structure of the mutant ovaries is due to defects within the ovariolar sheath. To determine how DWnt4 contributes to the structure of the sheath, we first examined its expression during ovarian morphogenesis.

### *DWnt4* Is Expressed in Apical Cells that Migrate to Form the Ovariolar Sheath Epithelium

Ovarian morphogenesis begins in the third larval instar and continues through pupal stages (King, 1970) (Figure 1G). At 2 hr after puparium formation (APF), the ovary is organized into four distinct cell populations. Germ cells and follicle cell precursors are located in the central region of the ovary (green and yellow, respectively). The terminal filament cells (red) are organized into stacks (Godt and Laski, 1995; King, 1970). The apical cell population (blue) begins to migrate basally between terminal filament stacks, secreting a thick basement membrane as it moves (King, 1970). Prior to and during their migration, the apical cells maintain close contact with each other and exhibit a roughly cuboidal morphology. By 24 hr APF, the migrating apical cells have separated the central region of the ovary into individual germaria and have begun to divide the basal cells (pink) into distinct clusters, called basal stalk precursors. By 36 hr APF, apical cell migration is complete, and the cells flatten to assume their final squamous morphology. By 48 hr APF, the first egg chambers have pinched off from the germaria.

DWnt4 is expressed throughout the apical cell population. At 2 hr APF, it is expressed in the apical cells (red) as they migrate basally between terminal filament stacks (green; Figure 1H). In ovaries isolated 16 hr APF, DWnt4-expressing cells have migrated between the terminal filaments and developing germaria (Figure 1I). By 18 hr APF, DWnt4-expressing cells have moved in between the individual basal stalk clusters (green; Figure 1J).

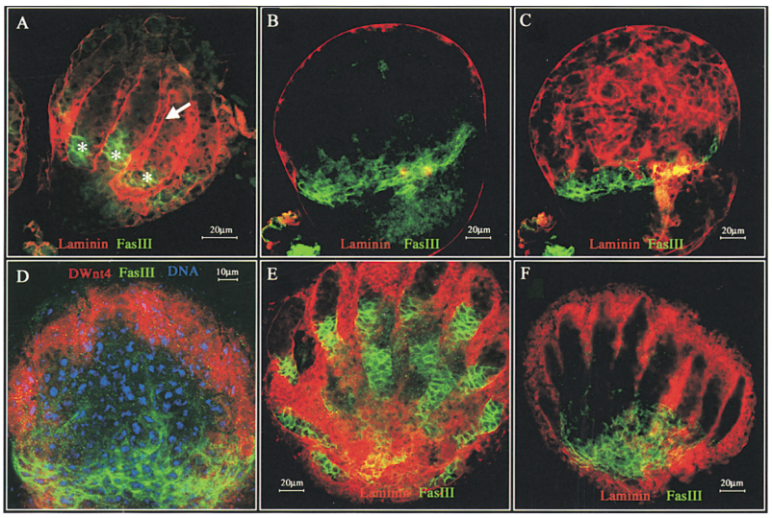


Figure 2. DWnt4 Is Required for Apical Cell Movement

(A and B) Ovaries at 18 hr APF stained with laminin (red) and FasIII (green). Basement membrane and basal cell clusters are indicated by arrows and asterisks, respectively. Focal planes lie in the interior of the ovary.
(A) Wild-type.
(B) *DWnt4* mutant.
(C) A peripheral optical section of the *DWnt4* mutant ovary shown in B.
(D) A *DWnt4* mutant ovary at 18 hr APF stained with anti-DWnt4 (red), FasIII (green), and Hoechst (blue, nuclei).
(E and F) Ovaries at 24 hr APF stained with laminin (red) and FasIII (green).
(E) Wild-type.
(F) *DWnt4* mutant.

These data show that DWnt4 is present throughout the apical cell population as these cells migrate to form the sheath.

### DWnt4 Is Required for Apical Cell Migration

The expression of DWnt4 in the apical cell population led us to examine these cells in *DWnt4* mutants. Two markers for the behavior of the apical cells are laminin, which is present in the basement membrane secreted by these cells as they migrate, and DWnt4 itself. In wild-type ovaries isolated 16 hr APF, laminin is concentrated in the basement membrane (Figure 2A). Lower levels of laminin are also observed surrounding individual apical cells. At this time, the apical population has migrated between clusters of basal cells.

In *DWnt4* mutant ovaries isolated 16 hr APF, laminin-secreting cells are absent from the interior of the ovary (Figure 2B). However, at the periphery of the ovary, laminin-secreting cells are apparent (Figure 2C), suggesting that the apical cell population is present and synthesizes basement membrane components. When the apical cells are visualized with the DWnt4 antibody, we also observe cells at the periphery of the ovary but not in the interior (Figure 2D). This contrasts with the wild-type, in which DWnt4-expressing cells separate clearly defined germaria (refer to Figure 1J). These data suggest that movement of the apical cells is disrupted in *DWnt4* mutants. However, since an epithelial sheath ultimately forms in mutant adults, we examined the apical cells 8 hr later. At 24 hr APF in wild-type ovaries, the basal stalks are well separated by the apical cells and are undergoing further morphogenesis to form columns (Figure 2E). In *DWnt4* mutants, sparse rows of laminin-secreting cells have begun to move into the central region of the ovary (Figure 2F) but have not begun to divide the basal cells. This indicates that limited cell movement does occur in *DWnt4* mutant ovaries. However, the paucity of moving cells and the delay in their movement results in epithelial sheaths that are not adequate to fully cover the mature adult ovariole.

Although DWnt4 could potentially serve as a polarizing cue for apical cells, two observations suggest that this is not the case: DWnt4 protein is present throughout the motile cell population and is therefore not polarized in its distribution, and the limited cell movement that does occur in *DWnt4* mutants is in the appropriate direction. An alternative role for DWnt4 is suggested by the observation that interruption of Wnt signaling in *Xenopus* inhibits the stabilization of lamellipodia at the leading edges of moving cells (Wallingford et al., 2000). Rather than providing a polarizing cue to specify direction of movement, Wnt signaling could stabilize the lamellipodia by promoting attachment between the cytoskeleton and the extracellular matrix. Since the role of focal adhesions is to provide such an anchor, we examined the localization of FAK as a marker of focal adhesions in migrating apical cells.

### FAK Accumulates in Spots in Apical Cells and Is Reduced in *DWnt4* Mutants

To examine the morphology of wild-type moving apical cells, we generated clones of cells labeled with a membrane-bound form of GFP to outline cell surfaces (Lee and Luo, 1999); a nuclear GFP was also expressed in these cells. The labeled cells in different regions within the apical population reveal that cells both at the leading front of the apical cell population (Figure 3A) and behind it (Figure 3B) extend processes and elongate in the general direction of movement. At 0–1 hr APF, the apical cells are just beginning to move in between the terminal filament stacks (Figure 3A).

We examined the focal adhesions that form in these cells using an antibody directed against *Drosophila* FAK as a marker (Fujimoto et al., 1999). At 0–1 hr APF, FAK has a predominantly filamentous appearance and colocalizes with F-actin within the cells and at the cell periphery (Figures 3A–3A″). The peripheral staining is most prominent at the cells' leading edges and sometimes appears as large spots (2–5 μ diameter; Figures 3A, 3A′, and 3C). This type of staining is most frequently observed at the leading front of the apical cell population. Throughout the apical cell population, we also observe smaller spots (submicron in diameter) of FAK staining (Figure 3B).

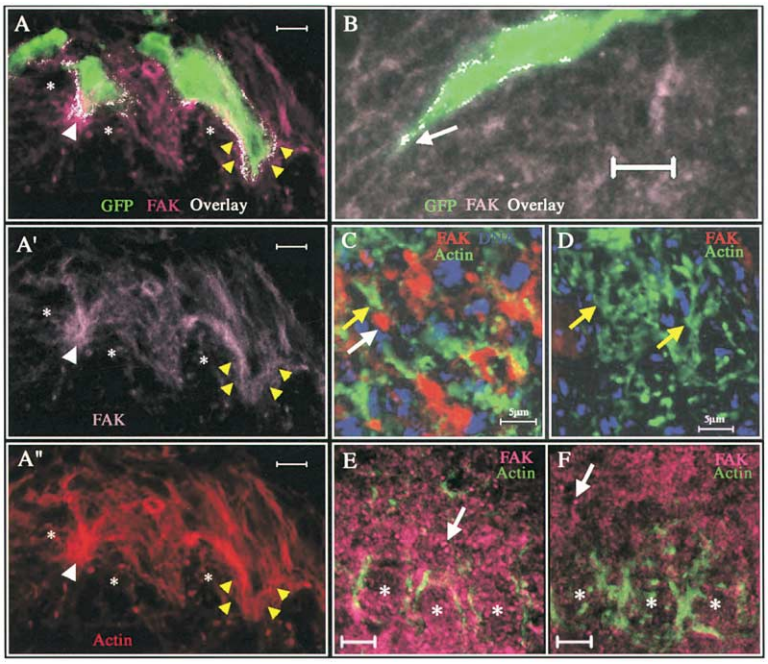


Figure 3. DWnt4 Regulates FAK in Apical Cells

(A and B) Wild-type apical cells at 0–1 hr APF labeled with CD8-GFP and a nuclear/cytoplasmic GFP (green), FAK (purple-pink), and the overlay of high levels of FAK and GFP (white).

(A–A″) Cells near the leading front of the apical population. Sometimes, the cells extend long processes (yellow arrowheads) that are lined with FAK; in other cells, the processes are shorter, and the high level of FAK appears as a large spot (white arrowhead). Asterisks indicate the position of the terminal filaments, marked by characteristic actin staining in a lower optical section.

(A) Overlay (white) between GFP (green) and high levels of FAK (pink). The FAK staining in the large spot and surrounding the long extension appears as many tiny puncta. However, when FAK is examined by itself (see [A′] and [3C] below), it appears simply as a large spot. We therefore refer to staining of this type as large spots.

(A′) FAK staining alone.

(A″) F-actin staining in the cells shown in A and A′.

(B) A wild-type cell located several cells away from the leading front of the population. The cell is elongated in the direction of its movement and has extended a slender projection. A small FAK spot is apparent at the very tip of the cell (arrow).

(C and D) Ovaries from wild-type (C) or *DWnt4* mutants (D) at 0–4 hr APF stained for FAK (red), F-actin (green), and DNA (blue). The optical sections shown here lie just above the terminal filament stacks, so the terminal filaments are not visible. White arrow indicates large FAK spot; yellow arrows indicate V-shaped actin structures. In this and all other panels below showing actin, the level of actin staining has been electronically reduced to prevent it from overwhelming the FAK staining. We believe that the large FAK spot shown in (C) is equivalent to the spot marked with a white arrowhead in (A) and (A′).

(E and F) Ovary at 2–4 hr APF from wild-type (E) or *DWnt4* mutant (F) stained for FAK (purple-pink) and F-actin (green). Arrows indicate small FAK spots; asterisks indicate terminal filament stacks. In all panels, apical is up, and the scale bar represents 5 μ.

These small spots are typically observed at the cell periphery. Their numbers increase in the early hours APF, so that, by 2–4 hr APF, the majority of FAK staining appear as small spots (Figure 3E).

To determine whether DWnt4 may affect cell motility by influencing focal adhesions, we compared FAK in wild-type and DWnt4 mutant ovaries. In wild-type cells located near the leading front of the apical population, FAK is present in large spots (Figure 3C). Many of these spots lie at the leading tips of the apical cells, as marked by V-shaped actin structures. In *DWnt4* mutant ovaries, the overall level of FAK is reduced, and the large FAK spots are not observed (Figure 3D). We do observe V-shaped actin structures, suggesting that the mutant cells are capable of extending cellular processes. At 2–4 hr APF, when FAK appears predominantly as small spots in wild-type (Figure 3E), the *DWnt4* mutant ovaries exhibit reduced, more diffuse FAK staining, but the small spots are not eliminated (Figure 3F).

The reduction of FAK may explain the failure of apical cell migration in DWnt4 mutants, since FAK is required for cell motility in other contexts (Furuta et al., 1995; Ilic et al., 1995, 1996). Our data indicate that DWnt4 signaling regulates directly or indirectly the accumulation of FAK. This may occur either through regulation of FAK levels, FAK subcellular localization, or both. We next sought to determine whether facilitation of movement and regulation of FAK occurs through the canonical Wnt signaling pathway, the planar polarity pathway, or a distinct signaling mechanism.

## DWnt4 Signals through DFz2 but Is TCF Independent

To determine whether DWnt4 signals through the canonical Wnt signaling pathway, we tested whether canonical pathway-specific components are required in migrating apical cells. D-TCF is a transcription factor that mediates Wg signaling but is not involved in planar polarity signaling (van de Wetering et al., 1997). Individuals lacking D-TCF die as embryos, due to loss of Wg signaling. Therefore, to examine its potential role in cell movement, we generated clones of cells that express an interfering form of this protein (TCFΔN) (van de Wetering et al., 1997). Although such clones in the wings and legs produce strong *wg*-like phenotypes (data not shown), TCFΔN does not interrupt apical cell movement. Apical cell clones expressing TCFΔN move between terminal filaments (Figure 4A), and FAK dots are readily observed within the cells (Figure 4B). This indicates that the effect of DWnt4 on apical cell movement is achieved via a TCF-independent mechanism.

DFz2 functions redundantly with Fz in canonical signaling but plays no role in planar polarity signaling (Boutros et al., 2000). *DFz2*$^{C1}$ homozygotes are adult viable but sterile (Chen and Struhl, 1999). The adult ovaries exhibit a "flopped" phenotype like those of *DWnt4*

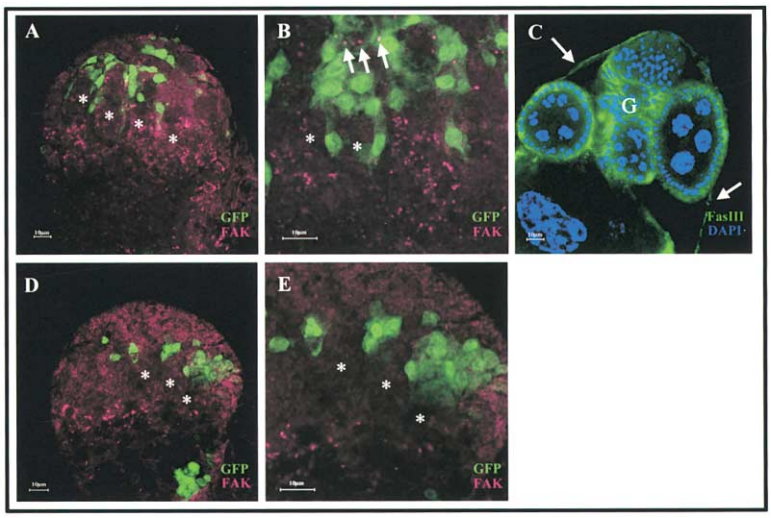


Figure 4. DWnt4 Signals through DFz2 but Is TCF Independent

(A and B) Clones of cells expressing TCFΔN were followed at 3–4 hr APF by the presence of coexpressed nuclear GFP (green; nuclear localization is not complete, so some cytoplasmic staining is also apparent) and stained with FAK (pink) and phalloidin (data not shown). Asterisks in (A) indicate the position of the terminal filaments; arrows in (B) indicate small FAK spots. TCFΔN expression with ptcGAL4 (see Figure 6) produced a similar, wild-type apical cell phenotype (data not shown).

(C) *DFz2$^{C1}$/Df(3L)DFz2* adult ovary. Staining with anti-FasIII (green; nuclei are blue) reveals the sheath (arrows) surrounding folded ovarioles (G, germarium). The female sterility associated with the *DFz2$^{C1}$* allele has been proposed to be the result of an unrelated mutation on the chromosome (Chen and Struhl, 1999). However, *DFz2$^{C1}$* mutants display an identical phenotype when either homozygous or placed over a deficiency. In addition, the *DFz2$^{C1}$* phenotype can be partially rescued by ubiquitous expression of a *DFz2* transgene. In the presence of the β-tub-DFz2 transgene, the percentage of abnormal ovarioles is reduced to 31% (63/202, compared to 80% in *DFz2* homozygotes; see Figure 6C), indicating that the mutant phenotype is due to loss of DFz2. Rescued females lay fertilized eggs; we presume that the lack of full rescue reflects inappropriate levels of expression from the transgene.

(D and E) DFz2-GPI-expressing cells (green) stained for FAK (pink) and F-actin (data not shown). Asterisks indicate terminal filaments.

mutants, suggesting that DFz2 functions as a DWnt4 receptor (Figure 4C; see also Figure 6C). We also examined clones of cells expressing a dominant-negative form of DFz2 (DFz2-GPI) (Zhang and Carthew, 1998). This form of DFz2 interrupts Wg signaling but does not produce planar polarity defects. DFz2-GPI-expressing apical cells are found clustered in the apical region of the ovary (Figure 4B), indicating that they are not capable of basal migration. Furthermore, when FAK is examined in cells expressing DFz2-GPI, spots of FAK are missing or severely reduced (Figure 4C).

These data indicate that DFz2 is required for apical cell migration and that it functions independently of TCF in this process. Furthermore, the fact that a *DFz2* mutation phenocopies the *DWnt4* apical cell migration phenotype suggests that DFz2 functions as a DWnt4 receptor in this process. Finally, the data suggest that DWnt4 is expressed by and signals to migrating apical cells, since expression of a truncated DFz2 protein intrinsically blocks migration of the cells.

### The DWnt4 Signaling Pathway Is Distinct from the Planar Polarity Pathway

Although neither *DWnt4* mutants nor *DFz2* mutants exhibit a planar polarity phenotype (data not shown), they could signal through the planar polarity pathway to regulate cell motility. To determine whether this is the case, we examined mutants that are defective in planar polarity signaling. Fz is the planar polarity receptor (Adler, 1992), but we see only minor ovarian defects in *fz* mutant adults (Figure 5A; see also Figure 6C). The *dsh* mutant allele *dsh$^{1}$* encodes a protein that transduces canonical signals but is defective in planar polarity signaling (Klingensmith et al., 1994; Thiesen et al., 1994). *dsh$^{1}$* mutants are fertile, but we noticed that they exhibit reduced fecundity. When we examined the ovaries from these females, we observed a *DWnt4*-like phenotype (Figure 5B; see also Figure 6C). The penetrance of the phenotype is reduced compared to that of *DWnt4*, presumably reflecting the fact that Dsh$^{1}$ retains some signaling ability (Boutros et al., 1998). The flopped ovarioles observed in *dsh$^{1}$* mutants suggest that Dsh is required for DWnt4 signaling in cell movement and that the *dsh$^{1}$* mutation disrupts DWnt4 signaling. To confirm that the flopped ovary phenotype of *dsh$^{1}$* mutants is due to failed cell movement, we examined FAK in *dsh$^{1}$* pupal ovaries (Figures 5D and 5D′) and compared them to wild-type (Figures 5C and 5C′) and *DFz2$^{C1}$* (Figures 5E and 5E′) mutants. FAK spots are visible in the wild-type apical cells but are reduced in both *dsh$^{1}$* and *DFz2$^{C1}$* mutants. This indicates that the Dsh$^{1}$ mutant protein can interfere with signaling through DFz2.

The phenotype of *dsh$^{1}$* and *DFz2$^{C1}$* suggested that the DWnt4 cell movement pathway uses a different receptor from the planar polarity pathway, but that it merges with planar polarity signaling at the level of Dsh. To address this possibility, we examined additional planar polarity mutants. A subset of the planar polarity mutants exhibit planar polarity defects in all tissues examined (referred to as "primary polarity genes"), while others affect polarity only in certain contexts (referred to as "secondary polarity genes") (Mlodzik, 2000). In addition to *fz* and *dsh*, *flamingo* (*fmi*), *diego* (*dgo*), *strabismus* (*stbm*), and *prickled* (*pk*) are included in the primary group, while *inturned* (*in*), *multiple wing hairs* (*mwh*), and *fuzzy* (*fy*) are included in the secondary group (Feiguin et al., 2001; Mlodzik, 2000). We have examined the adult phenotypes of amorphic or strongly hypomorphic alleles of each of these genes (Figure 6C). With the exception of *dsh$^{1}$*, none of these mutants displays significant disruption of ovarian morphology. This demonstrates that DWnt4, DFz2, and Dsh facilitate cell motility through a pathway that is independent of many of the planar polarity components.

In the wing, Dsh and Fmi proteins exhibit a polarized subcellular distribution (Shimada et al., 2001; Axelrod,

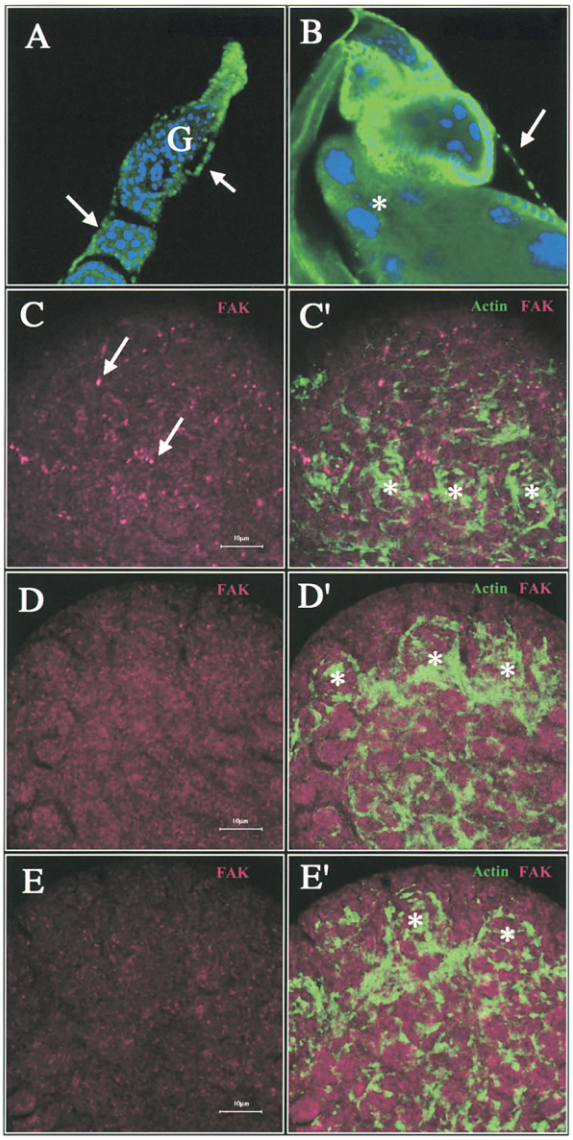


Figure 5. DWnt4 Signals through Dsh

(A) Adult ovary from $fz^{P21}/fz^{H51}$ mutant stained with an antibody to phosphorylated tyrosine, which marks cell membranes (arrows), and Hoechst (nuclei; G, germarium).

(B) $dsh^{1}/dsh^{VA153}$ mutant ovary stained with anti-FasIII (green) to mark the sheath (arrow) and Hoechst. Asterisk indicates an older egg chamber.

(C–E) Ovaries 2 hr APF stained with anti-FAK antibody (pink) and phalloidin (green). Asterisks indicate terminal filaments.

(C and C′) Wild-type. Arrows indicate FAK spots.

(D and D′) $dsh^{1}/dsh^{VA153}$.

(E and E′) $DFz2^{C1}$.

2001). This localization is necessary for the cells to be polarized along the proximal/distal axis. We therefore examined the distribution of these proteins in migrating apical cells to determine whether they are similarly polarized. Fmi is evenly distributed around the cell surface of apical cells (Figure 6A). Dsh is found on the cell membranes and also in the cytoplasm, but it is not polarized within the cells (Figure 6B). The nonpolarized distribution of Dsh and Fmi throughout the apical cell population suggests that they do not function in these cells to polarize the epithelium. Moreover, although overexpression of Dsh or Fmi disrupts epithelial polarization, similar overexpression has no discernible effect on apical cell movement (data not shown). These data indicate that the establishment of planar polarity and the promotion of cell motility occur through different cellular mechanisms.

### PKC Regulates Cell Motility and FAK in Apical Cells

Wnt signaling through PKC plays a role during vertebrate gastrulation (Kuhl et al., 2000; Winklbauer et al., 2001). To determine whether PKC may play a role in promoting apical cell migration, we generated clones expressing a pseudosubstrate of PKC (PKCi), which should effectively inhibit PKCs of different classes (Broughton et al., 1996) (Figure 6D). These clones resembled those expressing Dfz2-GPI, in that the apical cell clones were rarely observed between or beyond the terminal filament stacks. However, even when the clones have moved between the terminal filament stacks, their rounded morphology indicates a movement defect. In addition, FAK is reduced in these clones, although to a lesser extent than in DFz2-GPI clones. This suggested that PKC might be involved in promoting apical cell motility. To examine the effects of PKCi on a broader scale, we sought to express PKC throughout the apical cell population. The *ptc*GAL4 driver is active through much of the apical cell population (Figure 6F); using this driver to drive PKCi expression produces a more robust movement/FAK defect in apical cells (Figure 6E). PKCi expression in the wing did not perturb planar polarity or Wg-dependent margin specification (data not shown). These data suggest that PKC is required either with DWnt4 or in a parallel pathway to promote apical cell movement.

## Discussion

In the establishment of planar polarity and in cell movement, Wnt signals have been proposed to provide a polarizing cue through a noncanonical pathway (McEwen and Peifer, 2000; Tada and Smith, 2000; Wallingford et al., 2000). Our data allow us to relate DWnt4 to other factors that participate in cell motility and to compare directly the cell motility pathway to the planar polarity pathway. Our data reveal, first, that DWnt4 facilitates movement through a mechanism that includes FAK regulation and, second, that the pathway employed by DWnt4 is both TCF-independent and distinct from the planar polarity pathway.

### DWnt4 Facilitates Cell Movement through FAK

Since Wnt proteins are secreted, they are attractive candidates for directional signals. The failure of cell projections to stabilize in the absence of Wnt signaling in the frog has been proposed to reflect a loss of cell polarity. However, our data are inconsistent with a role for DWnt4 in providing a polarizing cue. DWnt4 is expressed throughout the population of moving cells and is required by these cells for their motility; it cannot therefore be the primary polarizing signal. The conclusion that Wnt signaling does not provide direction to cell movement is

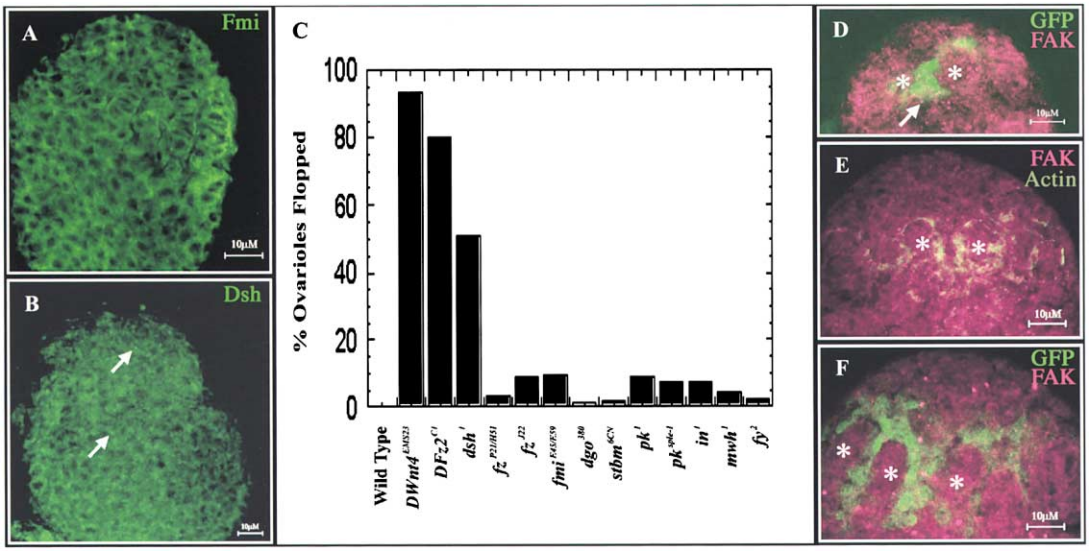


Figure 6. DWnt4 Does Not Signal through the Planar Polarity Pathway

(A and B) Wild-type ovaries at 0–4 hr APF.

(A) Fmi protein detected with anti-Fmi antibody.

(B) Dsh-GFP expressed under its own promoter/enhancers. In addition to cytoplasmic and cell surface staining, we observe small spots of Dsh-GFP (arrows). These do not colocalize with FAK (data not shown).

(C) Adult ovaries of the indicated genotypes were scored for the percentage of ovarioles that were flopped. A minimum of 100 ovarioles were scored for each genotype.

(D) A clone of PKCi-expressing cells, marked by coexpression of nuclear GFP (green), was stained for FAK (pink) and actin (data not shown; the position of the terminal filaments is indicated by asterisks). The arrow indicates FAK spots.

(E) ptcGAL4-UASPKCi stained for FAK (pink) and F-actin (green). The level of actin staining has been reduced electronically to improve the visibility of the FAK signal.

(F) The expression of ptcGAL4 in the ovary 0–4 hr APF, as marked by UAS-GFP; the position of the terminal filaments is indicated with asterisks, as marked by actin staining (data not shown). In all other tissues examined, *ptc* is expressed ubiquitously but is upregulated in those cells responding to *hh*. We assume that this is the case in the ovary as well.

also supported by the observation that loss of Wnt11-mediated movement during zebrafish gastrulation can be rescued by ubiquitous expression of Wnt11 (Heisenberg et al., 2000). Our data indicate that, rather than polarizing the cells, DWnt4 promotes the motility of apical cells through the regulation of focal complexes/adhesions.

Focal complexes in apical cells seem to change over the first few hours of pupal development as apical cell migration gets underway. FAK changes from primarily filamentous staining that is coincident with actin fibers at 0–1 hr APF to a more spotty appearance by 2–4 hr APF. The large spots of FAK that occur at the leading front of the population also become less pronounced over this period as more numerous smaller spots appear. The presence of FAK spots in moving apical cells and their position relative to actin, the leading edges, and the cell borders suggest that FAK is likely to participate in the regulation of motility and actin dynamics, as it does in cultured mammalian cells.

The analysis of FAK in mammalian cells suggests that the reduction of FAK in DWnt4 mutant cells is likely to impede their motility. FAK is activated upon localization to focal complexes and focal adhesions in mammalian cells. This leads to phosphorylation of multiple targets and ultimately to focal adhesion disassembly, allowing the cell to move forward (Igishi et al., 1999; Parsons et al., 2000; Turner, 2000). FAK is recruited to focal complexes when lamellipodia extend and establish contact with the extracellular matrix through integrin/matrix binding. Our data indicate that Wnt signaling is necessary for this recruitment to occur, either through regulation of FAK levels or through a more general regulation of focal adhesions. Interestingly, mouse Dsh localizes to focal adhesions in response to Wnt signaling in embryonic kidney cells (Torres and Nelson, 2000). Wnt signaling in these cells regulates the transition from mesenchymal morphology to epithelial morphology. This suggests that Wnt regulation of focal adhesions may be relevant for cell and tissue organization as well as for cell movement.

### DWnt4 Signal Transduction Is Not Mediated by TCF or the Planar Polarity Pathway

The requirement for DFz2 in apical cells initially suggested that DWnt4 facilitated movement through the canonical Wnt signaling pathway. However, analysis of TCFΔN-expressing clones indicates that this is not the case. This dominant-negative factor produces strong *wg*-like phenotypes in the appendages (van de Wetering et al., 1997) (data not shown) but has no effect on cell motility. In addition, the *DWnt4*-like phenotype exhibited by $dsh^1$ mutants is consistent with the involvement of a noncanonical pathway.

Similarity in phenotypes between $Dfz2^{C1}$ mutants and $dsh^1$ mutants has not been observed in other tissues. Loss of DFz2 has no impact on planar polarity signaling,

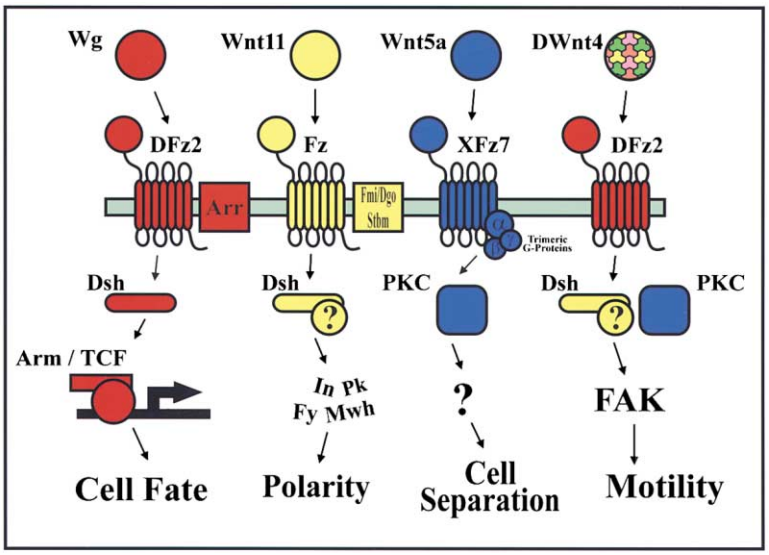


Figure 7. A Model of DWnt4 Signaling and Its Relationship to Other Wnt Pathways

Three distinct pathways have been proposed to transduce Wnt signals in different contexts: (1) the canonical Wg pathway (red), (2) the planar polarity pathway (yellow), which has also been proposed to mediate Wnt11 signaling in convergent extension, and (3) the Wnt/$Ca^{2+}$ pathway, which has been proposed to have Wnt5A as a ligand and which mediates cell separation during gastrulation (blue). Each of these pathways has membrane-associated components that appear to be specific to them. In addition, each appears to be distinct in the requirement of Dsh. The planar polarity pathway is disrupted by the *dsh*$^{1}$ mutation, whereas the canonical pathway is not. We have indicated this difference in the domains of Dsh that are important by coloring Dsh different colors in the two pathways. The Wnt/$Ca^{2+}$ pathway has been proposed to be Dsh independent. DWnt4 signaling may utilize components from each of these pathways. It uses the canonical pathway receptor, DFz2, and is disrupted by the planar polarity mutation of Dsh. This lesion in Dsh may inhibit interaction with a factor (indicated with a question mark) that connects Dsh activity to the cytoskeleton and that may therefore be involved in both planar polarity and cell motility. The recently identified DAAM1 protein may be a likely candidate for such a protein (Habas et al., 2001). However, several other planar polarity components, including those that function at the cell surface (e.g., Fmi and Dgo), are not required for DWnt4 signaling. Finally, our data implicate PKC signaling in the regulation of FAK, so it may be a part of the DWnt4 pathway.

whereas Fz is required for it (Boutros et al., 2000; Rulifson et al., 2000). Replacing the cysteine-rich ligand binding domain of DFz2 with that of Fz does not confer upon DFz2 the ability to alter planar polarity (Boutros et al., 2000). This has led to the suggestion that the intracellular domain of DFz2 can only engage the canonical pathway. Similarly, the *dsh*$^{1}$ mutation has been thought to interfere specifically with signaling from Fz. Our data show that neither of these suggestions is the case; DFz2 can engage a noncanonical, cell movement pathway given the appropriate ligand and cellular environment, and the Dsh$^{1}$ protein is unable to transduce a signal from DFz2 in this context. DWnt4 in apical cells therefore appears to recruit and use signaling components that have disparate activities in other tissues (Figure 7).

Although *dsh*$^{1}$ interferes with cell movement, the DWnt4 pathway is distinct from the planar polarity pathway. The first indication of this is that neither DWnt4 nor DFz2 mutants exhibit a planar polarity defect. However, we have noticed that ectopic expression of DWnt4 can mildly perturb planar polarity in the wing (E.D.C., unpublished data). The lack of mutant phenotype in planar polarity could therefore potentially be due to functional redundancies with other Wnt ligands. Alternatively, the ectopic-expression phenotype may be explained by interfering interactions between DWnt4 and Fz, such that DWnt4 binds Fz and prevents its signaling in the establishment of planar polarity. A similar phenomenon has been noted in the embryo, where DWnt4 can inhibit Wg signaling in the ventral epidermis (Gieseler et al., 1999). Since *DWnt4* mutants fail to show embryonic pattern defects, the ectopic expression phenotypes may not reflect endogenous DWnt4 functions.

Perhaps the strongest evidence that DWnt4 signaling and planar polarity signaling are different is the observation of normal ovarian morphology in several planar polarity mutants. The low-penetrance defects that have been observed in these mutants indicate that they only play a peripheral role, at best, in cell movement. Since none of the planar polarity mutants that we have examined other than *dsh*$^{1}$ exhibit significant defects in the ovariolar sheath, the process of determining polarity within an epithelial plane seems to be fundamentally distinct from Wnt facilitation of cell movement. This result is supported by the observation that neither Fmi nor Dsh protein is polarized within the moving apical cells, whereas the polarized localization of both is required in planar polarity (Shimada et al., 2001; Axelrod, 2001).

Although other factors regulate cell polarity and the actin cytoskeleton, such as the Rho family of small GTPases and the JNK pathway (Boutros et al., 1998; Paricio et al., 1999; Strutt et al., 1997), possible functional redundancies make their contribution to cell motility difficult to evaluate. However, since both cell polarity and cell motility involve regulation of the actin cytoskeleton, it seems likely that some of these factors may regulate both processes (see Figure 7).

If many of the core planar polarity components are not involved in DWnt4 signaling, what lies downstream of DWnt4/DFz2/Dsh in the regulation of cell motility? The negative effects of PKCi in our system suggest that the cell movement pathway may diverge from the planar polarity pathway to include regulation of PKC (see Figure 7). This is an interesting possibility, given the observation that PKC can regulate FAK activation and/or accumulation in fibroblasts (Mogi et al., 1995; Parsons et al., 2000). However, Wnt/PKC and Wnt/Dsh signaling in *Xenopus* appear to be required for different processes during gastrulation and thus to constitute distinct pathways (Winklbauer et al., 2001). Any analogy that may be drawn between PKC involvement in *Xenopus* and *Drosophila* apical cells assumes, however, that the PKC with which we have interfered is in the conventional, $Ca^{2+}$-dependent class. Since PKC pseudosubstrates

are not specific to a particular PKC class, we do not make this assumption.

Given the multiple PKC classes, their pleiotropic activities, and their ability to interact with many signaling pathways, further experiments are required to clarify the role of PKC in apical cells. However, our data indicate that Dsh and PKC share FAK/focal adhesions as a common target. Modulation of FAK/focal adhesions by a shared pathway or parallel pathways could potentially provide a mechanism for both the stabilization of cellular extensions and the regulation of cell-substratum adhesion.

## Do Wnt Proteins Regulate FAK Accumulation in Other Contexts?

Although we have examined Wnt regulation of FAK accumulation in one context, the near-ubiquitous expression of both FAK and Wnt family members suggests that regulation of FAK by Wnt proteins may be widespread. Focal adhesions and FAK are key regulators of cell movement and adhesion to the extracellular matrix in many contexts. They relay signals from the extracellular matrix for cell survival, migration, and proliferation. Our demonstration that Wnt signaling can regulate FAK suggests that Wnt proteins may exert some of their many effects through regulation of focal complexes and focal adhesions. This has implications not only for development but also in those adult tissues where Wnt signaling may regulate cell morphology, viability, migration, or proliferation.

## Experimental Procedures

### Fly Stocks

The following chromosomes were used: *Df(2L)RF*, *Df(2L)ade3*, and *Df(2L)DE* (Tiong and Nash, 1990); *y w hsflp; Sp/CyO; DFz2$^{C1}$ ri FRT2A/TM2* and the rescue stock *y w hsflp; Tub>DFz2/CyO; DFz2$^{C1}$ ri FRT 2A/TM2* (Chen and Struhl, 1999); *Df(3L)DFz2* (Bhanot et al., 1999); *dgo$^{380}$* (Feiguin et al., 2001); *stbm$^{6cn}$* (Wolff and Rubin, 1998); *cas-dsh-GFP*/TM6B (Axelrod, 2001); *fmi$^{E45}$* UAS-fmi/*fmi$^{E59}$ 1407 Gal4* (Shimada et al., 2001); *XX/y ras dsh$^{1}$*, *y w dsh$^{v26}$ FRT101/FM7*, *y w hsflp$^{22}$; Act5C>y+>GAL4*, *UAS-GFP$^{NLS}$/CyO*, *pk cn$^{1}$*, *pk$^{sple1}$*, *cp in$^{1}$*, *kni$^{ri-1}$ p$^{p}$*, *y; mwh$^{1}$*, *ptcGAL4*, *e22cGAL4*, and *cl fy$^{2}$ nub$^{2}$* (Bloomington Stock Center); *dsh$^{VA153}$ FRT18A/FM7* (from S. Cohen). All *dsh$^{1}$* flies were of the genotype *dsh$^{1}$/dsh$^{VA153\ or\ V26}$*.

Mutant chromosomes were rebalanced over either *FM7,Kr-GAL4 UAS-GFP* or *SM6a,TM6B* to allow mutant larvae and pupae to be distinguished from wild-type siblings. Expression in clones used the following: *UAS-DFz2GPI* (Zhang and Carthew, 1998), *UAS-TCFΔN* (dominant negative) (van de Wetering et al., 1997), *UAS-mCD8* (Lee and Luo, 1999), and *UAS-PKCi* (Broughton et al., 1996). The UAS-*DWnt4* line has been described (Gieseler et al., 1999).

Clones of cells expressing UAS constructs were generated by crossing a UAS line to flies of the genotype *y w hsflp$^{22}$; Act5C>y+>GAL4, UAS-GFP$^{NLS}$/CyO* (Ito et al., 1997); progeny were heat shocked for 1 hr at 32°C at second–third instar to enable GAL4 to be expressed. Clones were followed through the expression of *UAS-GFP*.

### Isolation of *DWnt4* Mutants

*DWnt4$^{EMS23}$* and *DWnt4$^{X1}$* were isolated following EMS mutagenesis of *cn bw sp* and *w$^{1118}$* males, respectively. *DWnt4$^{C1}$* was isolated following X-ray mutagenesis of *cn bw sp* males. All three mutations fail to compliment each other as well as three deficiencies that disrupt or remove the *DWnt4* locus: *Df(2L)DE*, *Df(2L)RF*, and *DF(2L)ade3* (Tiong and Nash, 1990). The coding region of each allele was sequenced from genomic DNA of hemizygous adults. Germline clones were analyzed using the FLP-DFS technique (Chou and Perrimon, 1996). The females bearing clones were fertile, and their progeny hatched, indicating that DWnt4 is not required in the germline.

In the course of sequencing the *DWnt4* alleles, we discovered an error in the original cDNA sequence. This error resulted in the predicted DWnt4 protein being 137 amino acids shorter than it actually is. We have confirmed this by comparing the sizes of the proteins produced by transfecting S2 cells with the full-length *DWnt4* cDNA and a cDNA with the 5′ end truncated to the original predicted start site (data not shown).

### Production of DWnt4 Antibody

To generate an antibody against DWnt4, the coding region from the originally reported start site was cloned into pET28a (Novagen) to produce a bacterially expressed His-tagged protein. The protein was purified on a Ni column and injected into rabbits (Pocono Rabbit Farm). Specificity of the antibody was evaluated by examining S2 cell-expressed proteins on Westerns and by examining expression in DWnt4 deficiency embryos (data not shown).

### Immunofluorescence

White prepupae were collected every 1–2 hr and aged for various times. Immunofluorescence was performed as described (White, 1998). The following antibodies were used: mouse monoclonal antibodies 4D9 anti-Engrailed/Invected and 7G10 anti-Fasciclin III, which were developed by C. Goodman and obtained from the Developmental Studies Hybridoma Bank, rabbit polyclonal anti-sera raised against Dlaminin (Fessler et al., 1987), guinea pig anti-Dfak56 #1562 (Fujimoto et al., 1999), and mouse anti-Fmi #74 (Usui et al., 1999). Rabbit polyclonal anti-sera against DWnt4 were generated in our laboratory as described. IgG purified anti-serum was diluted 1:50 and preabsorbed several times with S2 cell acetone powder prior to use. Alexa488-, Alexa556-, and Cy5-conjugated secondary antibodies against mouse, rabbit, and guinea pig were obtained from Molecular Probes. When desired, Alexa556-conjugated phalloidin was added to the diluted secondary antibodies to stain filamentous actin. Some samples were incubated with Hoechst 33342 dissolved in PBST to label cell nuclei.

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