Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways

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In *Drosophila,* planar cell polarity (PCP) signaling is mediated by the receptor Frizzled (Fz) and transduced by Dishevelled (Dsh). Wingless (Wg) signaling also requires Dsh and may utilize DFz2 as a receptor. Using a heterologous system, we show that Dsh is recruited selectively to the membrane by Fz but not DFz2, and this recruitment depends on the DEP domain but not the PDZ domain in Dsh. A mutation in the DEP domain impairs both membrane localization and the function of Dsh in PCP signaling, indicating that translocation is important for function. Further genetic and molecular analyses suggest that conserved domains in Dsh function differently during PCP and Wg signaling, and that divergent intracellular pathways are activated. We propose that Dsh has distinct roles in PCP and Wg signaling. The PCP signal may selectively result in focal Fz activation and asymmetric relocalization of Dsh to the membrane, where Dsh effects cytoskeletal reorganization to orient prehair initiation.

Drosophila Dishevelled (Dsh) was initially identified for its role in transducing the planar cell polarity (PCP; also known as tissue polarity) signal in developing imaginal disc epithelia (Adler 1992; Gubb 1993; Eaton 1997; Shulman et al. 1998). Dsh was shown subsequently to transduce an additional signal mediated by Wingless (Wg) (Klingensmith et al. 1994). We have investigated how Dsh functions in PCP signaling and whether it has the same or different function in Wg signaling.

PCP signaling controls the polarity of epithelial cells within a plane orthogonal to their apical–basal axis. One manifestation of this cellular polarity is the oriented organization of trichomes (cell hairs). In wild-type flies, the cell hair arising from each cell's distal vertex contributes to a parallel and specifically oriented array (Fig. 5A, below). Mutations in *dsh,* as well as additional genes including *frizzled* (*fz*), *prickle* (*pk*), *interned* (*in*), *fuzzy* (*fy*), *multiple wing hairs* (*mwh*), and others all disrupt the polarity of the trichomes. The resulting mutant phenotypes include swirls and distortions of the hair polarity pattern, and in some instances, more than one trichome

per cell (e.g., Fig. 5B, below). A putative signal transduction pathway has been proposed (Krasnow et al. 1995), which serves to polarize cells, allowing them to distinguish one side of the cell from the other, and to propagate this information from cell to cell. In this pathway, Fz, a seven-transmembrane protein (without apparent primary sequence homology to the G-protein-coupled receptors) acts as a receptor (Vinson et al. 1989; Adler et al. 1990), functioning upstream of Dsh. Dsh then antagonizes the activities of Fy, In (both novel transmembrane proteins; Park et al. 1996; Collier and Gubb 1997), and Mwh, which are in turn proposed to regulate the cytoskeletal apparatus responsible for control of PCP. Mutations in RhoA affect this process (Strutt et al. 1997), and experiments with dominant-negative mutants have implicated Cdc42 and Rac1 (Eaton et al. 1995, 1996) as additional effectors. Pk is proposed to function either in transmission of the signal to adjacent cells, or in interpretation of the directionality of the signal. Thus far, no ligand for the PCP pathway has been identified. Although a tentative signal transduction pathway has been proposed, the mechanism by which asymmetry is established in the responding cells is not understood.

In addition to transduction of the PCP signal, Dsh is required for Wg signal transduction (Perrimon and Mahowald 1987; Klingensmith et al. 1994; Miller and Moon

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1996). Wg, a member of the Wnt family, is a secreted glycoprotein that mediates a variety of cell fate choices throughout *Drosophila* development (Cadigan and Nusse 1997). The current model for Wg signaling proposes that in the absence of Wg signal, Zeste-white3 (Zw3, also known as Shaggy), a serine/threonine kinase homologous to GSK3, phosphorylates Armadillo (Arm, a homolog of β-catenin), destabilizing the cytoplasmic form of the protein. In the presence of Wg, the receptor acts through Dsh to antagonize Zw3 activity, and Arm is stabilized, resulting in its interaction with Pangolin (Pan, a TCF/Lef1-type transcription factor), forming a stable transcriptional regulator that can be seen in the nucleus. Based on cell-culture assays, *Drosophila Frizzled2* (*DFz2*) has been proposed to encode the Wg receptor (Bhanot et al. 1996), although confirmation awaits more definitive evidence. This observation also raises the possibility that another member of the Wnt family, of which four have been identified in *Drosophila,* could function as the PCP ligand to regulate Fz activity.

Wnt signaling appears to be highly conserved. A large family of Wnt proteins has been identified in various species, and many have been shown to act in signaling events (Cadigan and Nusse 1997). An extensive Fz family has also been identified that might function as receptors for Wnts (Orsulic and Peifer 1996), and mice and humans express multiple Dsh proteins (Sussman et al 1994; Klingensmith et al. 1996; Sokol et al. 1996; Tsang et al. 1996; Semenov and Snyder 1997). Wnts have been divided into at least two functional classes on the basis of their ability or failure to induce transformation of certain cultured mammalian cells (Wong et al. 1994). In *Xenopus,* members of the Wnt1 class have been shown to induce axis duplication and to function in a pathway similar to the Wg signaling pathway (Moon et al. 1997). In contrast, members of the Wnt5a class fail to induce axis duplication but, instead, alter morphogenetic movements during gastrulation (Moon et al. 1993, 1997). Whereas the Wnt1 class corresponds to *Drosophila* Wg in its signaling mechanism, it is unclear if the Wnt5a class corresponds to the PCP signaling mechanism.

Drosophila Dsh is a modular protein of unknown function that is well conserved in relation to its vertebrate homologs (Klingensmith et al. 1994; Thiesen et al. 1994). Alignment of family members reveals three conserved domains. The first, a DIX domain, is similar to a domain in murine Axin, a recently described modulator of the Wnt1 pathway (Zeng et al. 1997). The second contains a PDZ domain (Ponting et al. 1997); PDZ domains recognize and bind short motifs at the carboxyl termini of proteins (but may bind other motifs as well). PDZ domains can also form dimers. The third domain, called DEP, is conserved among a set of proteins that have in common the ability to regulate various GTPases, including both heterotrimeric G proteins and Ras-like small GTPases (Ponting and Bork 1996).

Here, we address how Dsh mediates the PCP signal and ask if this activity is distinct from its role in Wg signaling. We demonstrate that Dsh is recruited from the cytoplasm to the membrane and to filopodia by the PCP receptor, Fz, and that this relocalization is important for PCP signaling. The DEP domain is required for membrane localization of Dsh and is the site of a mutation that specifically disrupts PCP signaling. In contrast, the putative Wg receptor DFz2 fails to induce Dsh relocalization, and the DEP domain is not critical for Wg signaling. Finally, genetic and molecular assays suggest that Dsh makes distinct protein contacts in its two roles. We propose that membrane localization of Dsh mediates an asymmetric subcellular signal to direct a polarized response to the PCP signal.

Results

Localization of Dsh to the membrane and to filopodia in response to Fz

Of the known components of PCP and Wg signaling, *Drosophila* Dsh is thought to act most immediately downstream of the receptor. We were therefore interested in examining possible interactions between Dsh and the PCP receptor Fz and the putative Wg receptor DFz2. The sequences of these proteins suggested the possibility of a direct interaction. Dsh contains a PDZ domain, though it differs from the consensus by lacking a conserved basic residue at position 257 (Morais Cabral et al. 1996). Dfz2 has the consensus PDZ recognition peptide SXV at its carboxyl terminus (Bhanot et al. 1996), but Fz terminates with the sequence AXV (Vinson et al. 1989), which although similar, has not been shown to bind PDZ domains.

We chose to test whether either Fz or DFz2 could recruit Dsh to the membrane, perhaps via the PDZ motif, by introducing these components into a heterologous system that is rapid, allows controlled expression of multiple proteins, and is amenable to analyzing the intracellular distribution of proteins. mRNAs encoding Fz or DFz2 and a fusion of Dsh to green fluorescent protein (Dsh–GFP) were synthesized in vitro and injected into *Xenopus* embryos at the four-cell stage. Animal caps from stage 9 embryos were dissected to reveal the blastocoelar cells, which were examined by confocal microscopy as described previously (Yang-Snyder et al. 1996).

We first examined the relationship between Dsh–GFP and Fz. Dsh–GFP, when introduced alone, is observed predominantly in association with cytoplasmic vesicles (Fig. 1A). By comparison, in *Drosophila,* Dsh is observed to be cytoplasmic but shows a vesicular pattern in late-stage embryos (Yanagawa et al. 1995). In third instar imaginal discs, Dsh is also cytoplasmic, but pupal-stage discs have not been examined. We have noted previously that upon overexpression in cultured *Drosophila* S2 cells, Dsh also localizes to cytoplasmic vesicles (Axelrod et al. 1996). When Fz is expressed simultaneously with Dsh–GFP, Dsh–GFP shows a qualitative redistribution to the membrane or cell cortex (Fig. 1B–D). Under these conditions, we also noticed localization of Dsh–GFP to filopodia present on the blastocoelar (free) surfaces of the animal cap cells (Fig. 1E–G). Staining with phalloidin (and Dsh–GFP) revealed that the filopodia contain filamentous actin (Fig. 1G′). It is interesting to note that
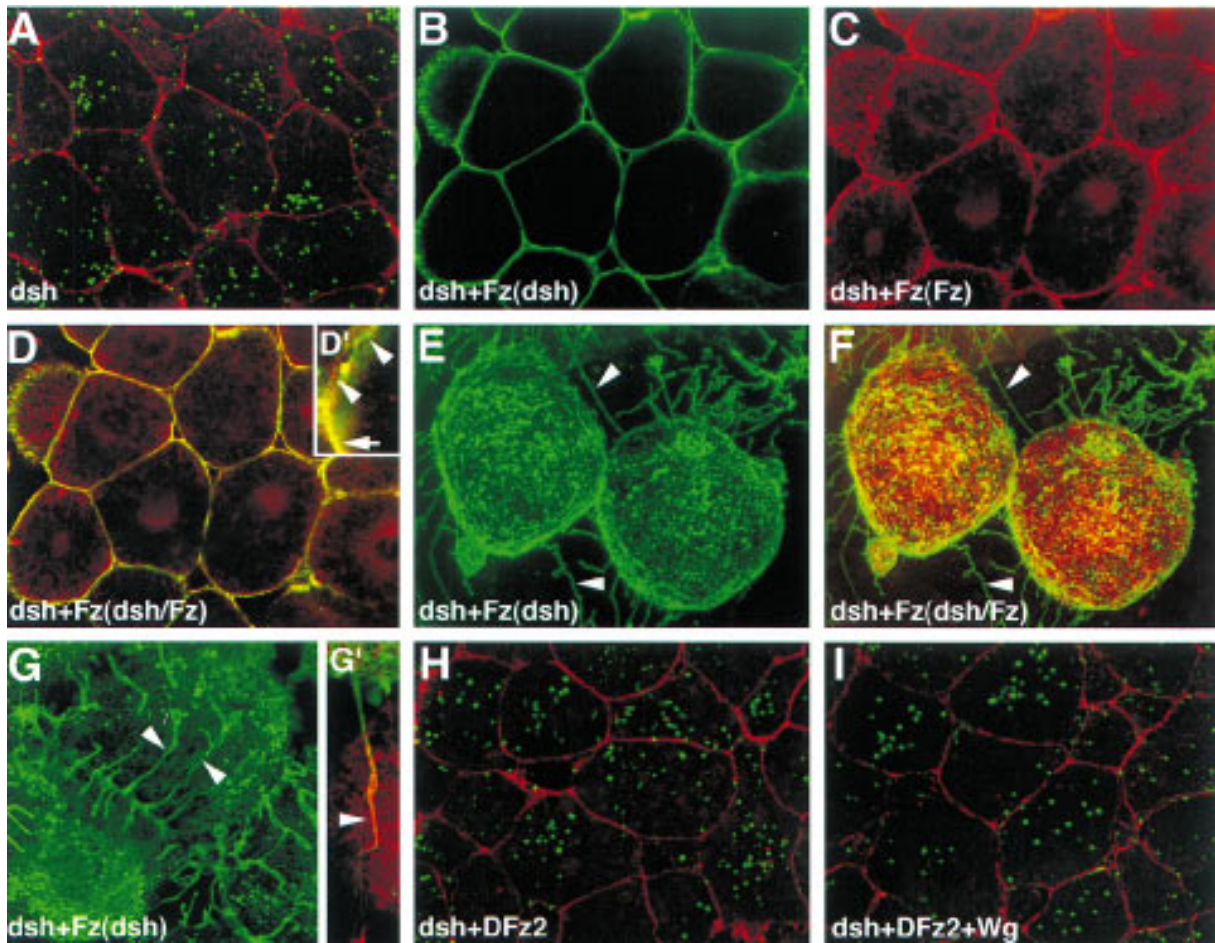
Figure 1. Dsh–GFP localizes to the membrane and filopodia in response to Fz in *Xenopus* animal cap cells. (*A*) Dsh–GFP (green) localizes in the cytoplasm in an apparent association with intracellular vesicles (Texas Red phalloidin labels the cell cortex). (*B*) In response to Fz, Dsh–GFP redistributes to the plasma membrane. (*C*) Fz localizes predominantly to the plasma membrane in the presence (shown) or absence (not shown) of Dsh–GFP. (*D, D′*) Dsh–GFP colocalizes with Fz at the plasma membrane. Yellow staining represents colocalization of Dsh–GFP (green from *B*) and Fz (red from *C*) and is marked by an arrow in D′. However, regions of the plasma membrane are also stained only by Dsh–GFP or Fz, demonstrating that the colocalization of Dsh–GFP and Fz is not absolute (arrowheads in *D′*). (*E–G*) In response to Fz, Dsh–GFP also accumulates in filopodia that extend from the free surface of the animal cap cells (arrowheads). These filopodia contain actin (stained with phalloidin; arrowhead in *G′*) but lack Fz (note lack of yellow staining in filopodia in *F*). (*H,I*) In contrast to the effects of Fz on the localization of Dsh–GFP, neither DFz2 (*H*) nor the combination of DFz2 and Wg (*I*) results in a change in the localization of Dsh–GFP (Dsh–GFP = green). Texas Red phalloidin was used to visualize cell outlines in *A, H,* and *I,* and the filopodium in *G′*.

although the filopodia stain with Dsh–GFP, little or no Fz localizes there, and that at the cell cortex, the Fz and Dsh–GFP show imperfect colocalization (Fig. 1D′). Fz staining is localized predominantly to the plasma membrane, and to a lesser extent to intracellular membranes (probably ER and/or Golgi) in these cells (Fig. 1C,F). This suggests that, whereas Fz may induce localization of Dsh–GFP to the membrane and filopodia, it may do so by a mechanism other than direct binding.

Dsh domain requirements for Fz-dependent relocalization

To test whether recruitment of Dsh–GFP to the membrane by Fz depends on an interaction between the Dsh PDZ domain and the carboxy-terminal AXV of Fz, we used the *Xenopus* animal cap assay to test for localization of a Dsh construct from which the PDZ domain and the basic domain had been deleted [Dsh(ΔbPDZ)–GFP, Fig. 6, below]. Strikingly, in the presence of Fz, Dsh(ΔbPDZ)–GFP localizes to the membrane and to filopodia in a manner indistinguishable from intact Dsh–GFP (Fig. 2A,B). Similarly, a Fz lacking the carboxy-terminal AXV retains its ability to induce membrane localization of Dsh–GFP (not shown). These results argue that recruitment of Dsh–GFP to the membrane occurs by a mechanism not requiring the Dsh PDZ domain or a potential PDZ target peptide in Fz.

Because the PDZ domain appears to play no role in the relocalization of Dsh in response to Fz, we tested the

requirements for the other conserved domains in this response. When a construct carrying a deletion of the DIX domain (Fig. 6, below) was tested as above, we noted that in the absence of exogenous Fz protein, Dsh(ΔDIX)–GFP did not associate with cytoplasmic vesicles, but instead was distributed uniformly throughout the cytoplasm, and some appeared to localize at the membrane (Fig. 2C). In the presence of Fz, Dsh(ΔDIX)–GFP relocalized to the membrane and to filopodia as does the wild-type protein (Fig. 2D). We conclude that the DIX domain contains sequences necessary to anchor Dsh to the cytoplasmic vesicles. The nature and significance of these vesicles are unknown, though they appear not to be insoluble, improperly folded Dsh, as the Dsh–GFP fluoresces and can be mobilized to the membrane in the presence of Fz.

We next tested the requirement for the DEP domain. Dsh(ΔDEP)–GFP (Fig. 6, below) is seen in association with cytoplasmic vesicles in the absence of Fz, and is indistinguishable from wild-type (Fig 2E). However, when Fz is coexpressed, Dsh(ΔDEP)–GFP fails to relocalize to the membrane or to filopodia, but instead remains associated with the cytoplasmic vesicles (Fig. 2F). Therefore, the DEP domain is required for the relocalization of Dsh in response to Fz. Finally, we asked whether the DEP domain is sufficient to cause relocalization of Dsh to the membrane. Dsh(DEP+) (Fig. 6, below) is cytoplasmic in the absence of Fz, as expected, because it lacks the DIX domain (Fig. 2G). In the presence of Fz, Dsh(DEP+) relocalizes to the membrane (Fig. 2H), demonstrating that the DEP domain is sufficient to relocalize Dsh in response to Fz.

dsh[1], a mutant that is functional for Wg but not PCP signaling

dsh[1] is an allele that behaves as a genetic null for PCP signaling, but is fully functional for Wg signaling (Perrimon and Mahowald 1987). dsh[1] might encode a promoter or enhancer mutation, such that levels of Dsh might be produced that are sufficient to sustain Wg signaling, but not PCP signaling. In contrast, we hypothesized that dsh[1] might encode a structural mutation, in-
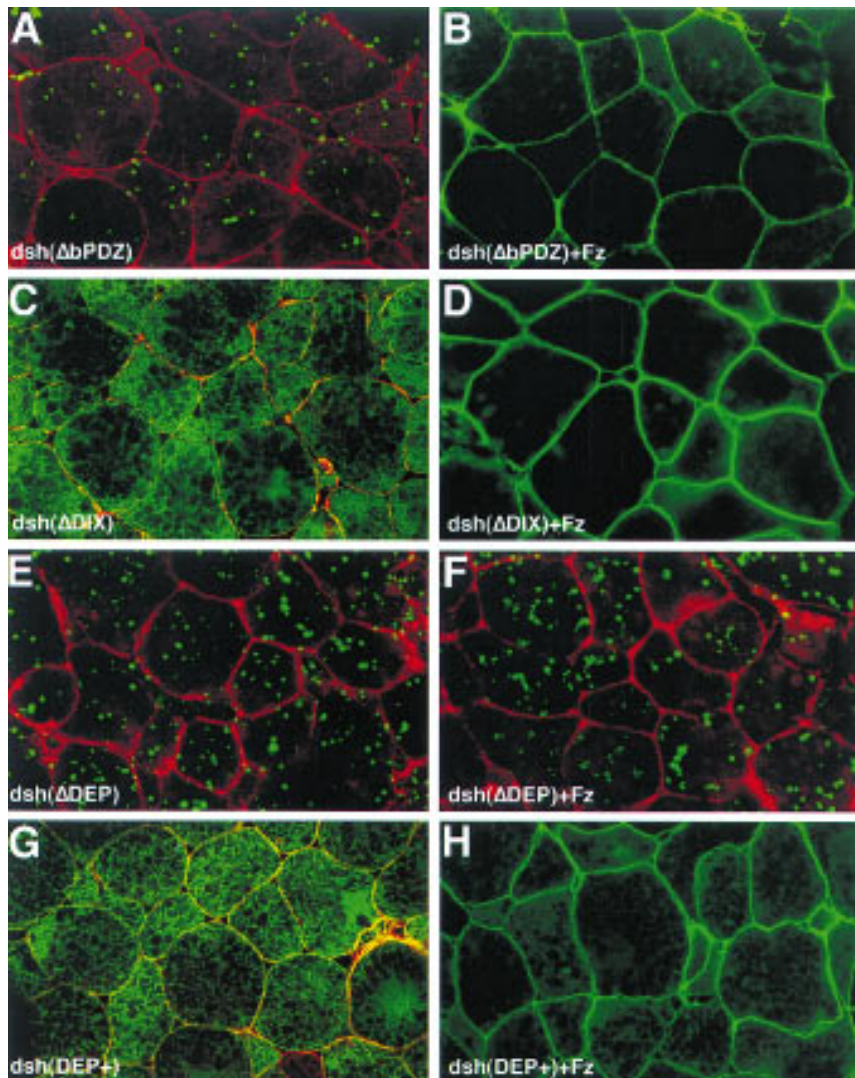

Figure 2. Analysis of Dsh domains required for Fz-dependent relocalization of Dsh in *Xenopus* animal cap cells. (*A*) Dsh(ΔbPDZ)–GFP localizes to the cytoplasm in a punctate pattern. (*B*) In response to Fz, Dsh(ΔbPDZ)–GFP relocalizes to the plasma membrane (shown) and is also present in filopodia (not shown). (*C*) Dsh(ΔDIX)–GFP does not display a punctate pattern and instead is distributed diffusely throughout the cytoplasm (but is excluded from yolk granules). (*D*) In response to Fz, Dsh(ΔDIX)–GFP relocalizes to the plasma membrane (shown) and is also present in filopodia (not shown). (*E*) Dsh(ΔDEP)–GFP localizes to the cytoplasm in a punctate pattern. (*F*) Dsh(ΔDEP)–GFP, however, does not relocalize to the plasma membrane in response to Fz and displays a punctate pattern similar to that seen in the absence of Fz. (*G*) Dsh(DEP+) is diffusely cytoplasmic in the absence of Fz, and is sufficient to promote membrane localization in the presence of Fz (*H*). Texas Red phalloidin was used to visualize cell outlines in *A, C, E,* and *G.*

dicating a differential activity of Dsh in the two pathways. We therefore cloned the *dsh¹* allele, and found a single amino acid substitution of M for K at position 417 (Fig. 3). This residue is conserved among the *dsh* homologs so far isolated, and is conserved moderately among the recognized DEP domains. To verify that this substitution confers the *dsh¹* phenotype, this mutation was created in a wild-type genomic DNA fragment, P-element transformants were created, and these were used to rescue *dsh* null mutant flies. The rescued flies were viable and had the *dsh¹* phenotype; this was independent of the copy number of the transforming gene, from one to three copies (not shown). Thus, a single amino acid substitution in the DEP domain of Dsh confers a loss of function for PCP signaling, yet the mutant protein is functional for Wg signaling.

The dsh¹ mutation affects Fz-dependent localization

Because the *dsh¹* mutation maps to the DEP domain, and the DEP domain is required for Dsh localization in response to Fz, we hypothesized that the *dsh¹* mutation may disrupt membrane localization. We therefore examined the behavior of *dsh¹* in the *Xenopus* assay. Expressed alone, Dsh¹–GFP associates with cytoplasmic vesicles (Fig. 4C). In the presence of Fz, Dsh1-GFP localizes to the cell cortex, but the cortical Dsh¹–GFP remains vesicular, rather that distributing evenly as does the wild-type protein (Fig. 4). We propose that the *dsh¹* mutation allows translocation, but impairs the ability of Dsh¹ to associate with its target at the membrane, and that this altered membrane interaction diminishes the ability of Dsh¹ to function in PCP signaling.

DFz2 fails to induce membrane localization of Dsh

Because DFz2 has been proposed to serve as the receptor for Wg, we tested whether it could also induce membrane localization of Dsh. In contrast to Fz, we found that DFz2 fails to recruit Dsh–GFP to the membrane (Fig. 1H). Although we were unable to verify that wild-type DFz2 was expressed, a myc-tagged DFz2 was expressed in a pattern similar to that seen for Fz, and it also failed to induce Dsh–GFP membrane localization (not shown). One explanation for the failure of DFz2 to recruit Dsh is that an endogenous ligand is present and required to activate Fz, but no endogenous ligand for DFz2 is present. To rule out this possibility, we expressed Wg, a ligand for DFz2, together with DFz2 and

Dsh–GFP. Wg failed to promote membrane localization of Dsh–GFP (Fig. 1I), although it was active as evidenced by its ability to induce *Xnr3* expression and an ectopic dorsal axis as reported previously (data not shown) (Chakrabarti et al. 1992). We also verified that DFz2 was functional. Under identical conditions, DFz2 activated *Xnr3* expression to levels comparable to those seen with Wg or Dsh (not shown). These data indicate that even in the presence of a functional DFz2 and a functional ligand, Dsh–GFP is not recruited to the membrane.

Dsh domains behave differently in PCP and Wg signaling

The *dsh¹* mutation is a structural mutation in the DEP domain affecting one but not the other pathway in which Dsh participates. Because the DEP domain is required for Fz-specific relocalization of Dsh, this suggests that the DEP domain of Dsh may be making specific contacts and functioning differently in the two pathways. To test this hypothesis more specifically, and to extend it to the other conserved Dsh domains, we created a series of Dsh deletion constructs and tested them for function in the Wg and PCP signaling pathways.

To test function in the PCP pathway, constructs were expressed ubiquitously at 24 hr after pupariation (AP), when PCP signaling is known to occur (Krasnow and Adler 1994). We have demonstrated that overexpression of full-length Dsh causes a dominant PCP phenotype (Fig. 5C). This phenotype, although reminiscent of the loss-of-function *dsh¹* phenotype, is distinct because overexpression of Dsh suppresses the *dsh¹* phenotype (Fig. 5H), and because a distinct and stereotypical pattern of polarity is seen (Fig. 5B,C). Similarly, deletion constructs that showed a phenotype were determined to be functional or to be dominant negative by expressing them in a *dsh¹* background, and in some cases, simultaneously with full-length Dsh. Examples of the data are shown in Figure 5A–H. Expression of only the DEP domain produces a polarity phenotype that is similar to *dsh¹,* and was demonstrated to be dominant negative. Dsh(ΔbPDZ) and Dsh(ΔDEP+) behaved similarly, whereas Dsh(ΔDIX) produced only a weak phenotype in wild type that could not be further characterized (Fig. 5G).

Function was tested in the Wg pathway by asking whether these constructs could either rescue *dsh* mutant embryos or cause dominant-negative effects in phenotypically wild-type embryos. To do so, mRNAs for the various constructs were created, and these were injected into embryos that were either mutant for *dsh* or phenotypically wild type. These were generated from the same cross (because the *dsh* maternal effect is paternally rescuable) and could be distinguished by the presence or absence of the cuticle marker *shaven baby* (*svb*). In this way, the ability to rescue the mutant phenotype, as well as the ability to function as a dominant negative in a phenotypically wild-type background, could be assessed by scoring the cuticle phenotype (Klingensmith et al. 1996). Examples of the assay results are shown in Figure 5I–N.
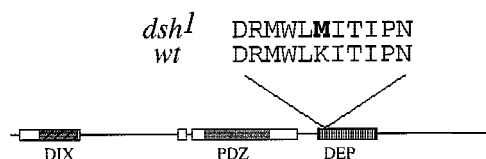

dsh¹ DRMWL**M**ITIPN
wt   DRMWLKITIPN

DIX   PDZ   DEP

Figure 3. Sequence of the *dsh¹* mutant allele. The *dsh¹* allele carries a Lys → Met mutation in the DEP domain, at position 417. The conserved Dsh domains are boxed, and the recognized motifs are shaded.
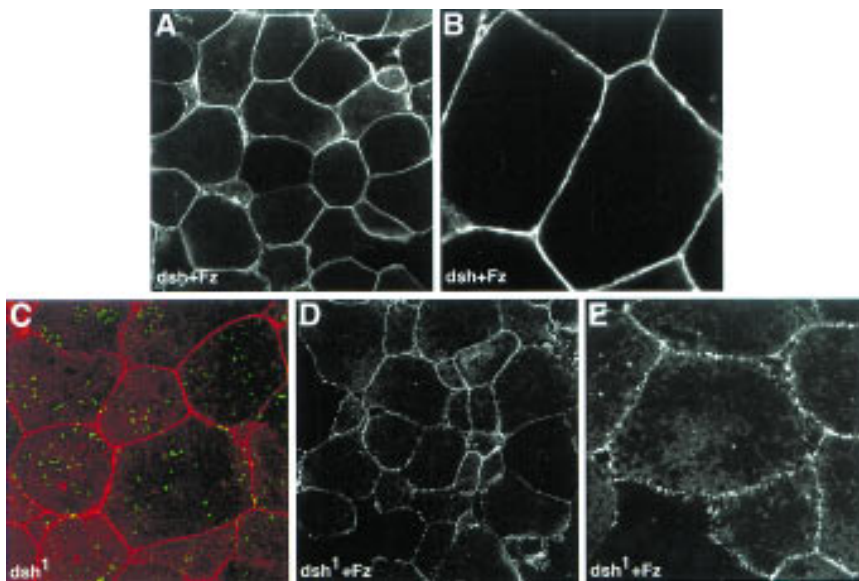
Figure 4. The *dsh¹* mutation affects Fz-dependent localization. (*A,B*) In response to Fz, Dsh–GFP redistributes to the plasma membrane in a diffuse pattern. (*D,E*) In contrast, Dsh¹–GFP redistributes to the membrane but remains associated with vesicles at the cell periphery. This effect was seen at all ratios of *dsh* to *fz* RNAs; the experiment shown used a ratio of 2:1. (*C*) In the absence of Fz, Dsh¹–GFP is vesicular, and is indistinguishable from wild-type Dsh–GFP.

The structures of the constructs tested, and the results of the structure/function analyses, are shown in Figure 6. A striking discordance between the activities of the various constructs in the two assays is noticeable immediately. For example, the individual domains Dsh(DIX) and Dsh(bPDZ), are each dominant negative for Wg sig-
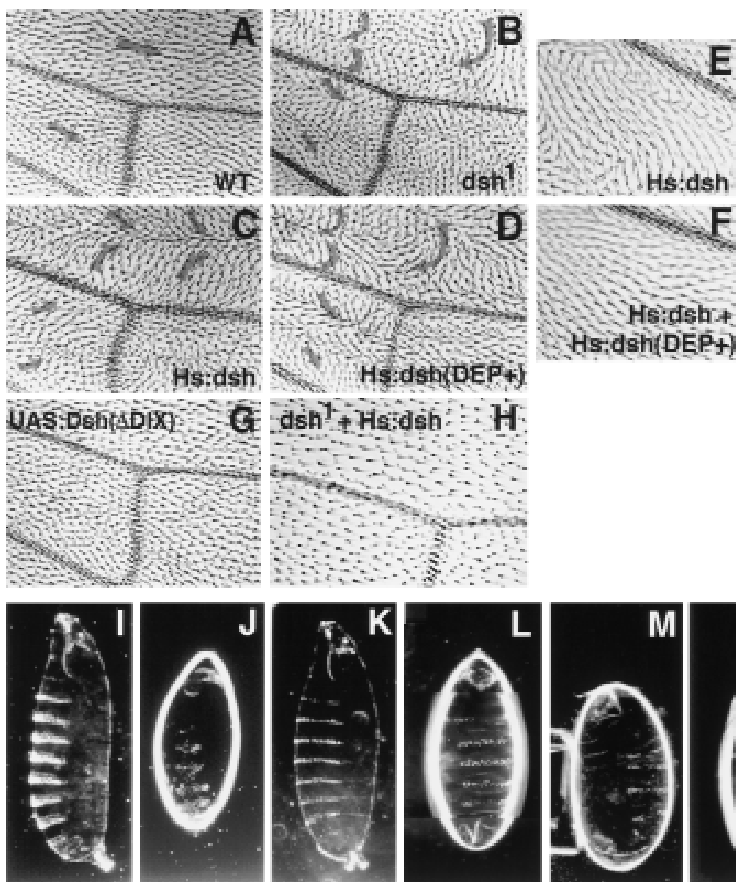

Figure 5. Dsh structure function analyses. Analysis of function in PCP signaling. Regions of wings from (*A*) wild type, (*B*) *dsh¹*, (*C*) *T8Hs:dsh/+* heat-shocked for 1 hr at 24 hr AP, (*D*) *Hs:dsh(DEP+)* heat-shocked for 2.5 hr at 24 hr AP. Note that the *Hs:dsh(DEP+)* wing resembles the *dsh¹* wing, rather than the *Hs:dsh* wing. A different region of a *T15Hs:dsh/+* wing shows a pattern (*E*) that is suppressed by simultaneous expression of *Hs:dsh(DEP+)* (*F*). (The flies in *E* and *F* were heat-shocked for 1 hr at 24 hr AP. A slightly higher magnification is shown.) Expression of Dsh(DEP+) in a *dsh¹* mutant failed to alter the *dsh¹* phenotype (not shown). By these criteria, Dsh(DEP+) behaves as a dominant negative. Expression of wild-type Dsh suppresses the *dsh¹* phenotype (*H;* the wing is marked with yellow; thus the hairs are finer and have less contrast. A slightly higher magnification is shown). Dsh(ΔDIX) produced only a weak phenotype when expressed by UAS and a range of GAL4 drivers (Hs:GAL4 is shown, *G*). Analysis of function in Wg signaling. (*I*) A wild-type cuticle; (*J*) a *svb, dsh* cuticle. The *dsh* mutant cuticle shows shortening, fusion of denticle bands, and absence of the head skeleton and filzkorper characteristic of mutants in the Wg pathway. Injection of wild type mRNA into the mutant embryos results in complete rescue of segmentation, the head skeleton and the filzkörper (*K*). Cuticles injected with Dsh(ΔbPDZ) are identical. Similarly, injection of partially functional constructs partially rescues segmentation, the head skeleton and the filzkörper to varying extents; e.g., (*L*) Dsh(Δ*EP+) and (*M*) Dsh(ΔDIX). Conversely, injection of dominant-negative constructs causes wild-type embryos to show a segmentation phenotype and loss of the head skeleton and filzkörper similar to the mutant embryos; (*N*) Dsh(DIX) and Dsh(bPDZ) (not shown).

Figure 6. Structures of the Dsh constructs. The conserved domains are as in Fig. 3, and the nonconserved basic domain is indicated (b). Results of the structure–function analyses are shown in semiquantitative form. Embryos that hatched are indicated; (DN) dominant negative. A detailed summary is found in Materials and Methods. (GFP) Some of the same constructs were fused to GFP at their carboxyl termini and used in the animal cap assays.

	DIX	b	PDZ	DEP		PCP assay	Wg assay
Dsh						+++	+++ (hatch)
Dsh(ΔDIX)					(GFP)	+/-	++
Dsh(ΔbPDZ)					(GFP)	DN	+++ (hatch)
Dsh(ΔDEP+)						DN	-
Dsh(Δ*EP+)							++
Dsh(DIX)					—	-	DN
Dsh(bPDZ)						-	DN
Dsh(DEP+)					(GFP)	DN	-
Dsh(ΔDEP)					(GFP)		

naling but have no effect on PCP signaling. The simplest explanation for these observations is that the various domains of Dsh are contacting different partners in the two pathways. The behavior of the DEP domain in the *Xenopus* assay is consistent with this hypothesis.

Several of these results are germane specifically to the function of Dsh in PCP signaling. Deletion of most of the DEP domain [Dsh(Δ*EP+)] leaves Wg signaling largely intact; however, in PCP signaling, a dominant-negative activity results from loss of the DEP domain [Dsh(ΔDEP+)]. Conversely, expression of just the DEP domain also had a dominant-negative PCP but not Wg phenotype [Dsh(DEP+)]. This suggests a critical role for the DEP domain in PCP signaling that is not shared by the Wg pathway.

The DIX domain anchors Dsh to cytoplasmic vesicles, and its deletion reduces function in both pathways. Anchoring may therefore be important for both pathways.

Also of note, deletion of the PDZ domain [Dsh(ΔbPDZ)] resulted in a construct that is functional for Wg signaling but dominant negative for PCP signaling. It must therefore play a significant role in PCP signaling, but a potential role in Wg signaling may only be significant when the protein is expressed at wild-type levels (see below).

Wg and PCP signaling pathways require different components downstream of Dsh

To determine whether Dsh plays the same or different roles in PCP and Wg signaling, we evaluated two distinct models. In the first model, Dsh has similar functions in the two pathways, and may use a common set of effectors. In such a model, Dsh, Zw3, and Arm might function as a signaling cassette in a manner analogous to the functions of Ras and Raf. In an alternate model, Dsh has different functions in the two pathways, and interacts directly with different downstream components.

Because overexpression of either Dsh or Fz at 24 hr AP produces dominant PCP phenotypes (Krasnow and Adler 1994; Adler et al. 1997; see below), we asked whether overexpression of Zw3 or Arm might also affect planar polarity. Overexpression of Zw3, or an activated Arm protein (Arm[S10]; Pai et al. 1997) at 24 hr AP failed to produce any effect on PCP (Fig. 7A,B).

We then asked whether loss of function had any effect

on PCP. Because clones of cells carrying null mutations in *arm* cannot be recovered in the adult (Peifer et al. 1991; Fig. 7C), we selected a hypomorphic *arm* allele, *arm[H8.6]*, that is strongly mutant for Wg signaling (Klingensmith et al. 1989). Clones mutant for *arm[H8.6]* (and marked with *f[36a]*) showed no disturbance in planar polarity (Fig. 7E), suggesting that Arm plays no role in this signaling event.

Clones mutant for *zw3* alter cell fate, causing epithelial cells in the wing to adopt a neural fate and produce tufts of bristles (Simpson et al. 1988; Fig. 7C). We therefore generated clones that were doubly mutant for *zw3* and *scute* (*sc*). *sc* mutants are unable to adopt the neural fate such that *sc zw3*-mutant cells remain epithelial. *zw3 sc* double-mutant clones (marked with *f[36a]*) show no polarity disturbance, suggesting that Zw3 is not involved in PCP signaling (Fig. 7F).

Although a null *arm* allele cannot be used for this analysis, and an activated form of *zw3* is not available, we believe these experiments argue strongly against a role for these genes in planar polarity signaling. Similarly, since mutations in the planar polarity genes *fz, pk,*
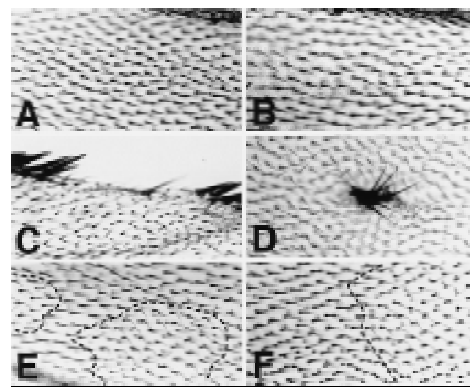

Figure 7. Arm and Zw3 have no role in PCP signaling. Wings from flies in which Arm[S10] (*A*) or Zw3 (*B*) were overexpressed show a wild-type polarity phenotype. Clones of *arm[H8.6]* (*C*) or *zw3* (*D*) mutant cells marked with *f[36a]* produce wing margin nicks and tufts of bristles, respectively. The *zw3* mutant cells appear to cause polarity distortions in the surrounding wild-type tissue, probably by grossly distorting the architecture of the epithelium. However, cells in *arm f[36a]* mutant clones in the interior of the wing produce hairs of wild-type polarity (*E*), as do cells in *sc[B57] zw3 f[36a]*-mutant clones (*F*).

in, fy, and *mwh* produce viable adults, there is no evidence of a role for these genes in Wg signaling. Thus, the known components functioning downstream of Dsh in both pathways are distinct.

Reciprocal titration of PCP and Wg signaling

The above analyses suggest that Dsh has different interactions in PCP and Wg signaling. This model predicts an additional genetic behavior. If Wg and planar polarity signaling utilize Dsh in a common fashion, then ectopic activation of one pathway should be able to cross-activate the other by promiscuously activating Dsh. In contrast, if each pathway utilizes Dsh in a distinct fashion, then ectopic activation might sequester Dsh in pathway-specific complexes, rendering it unavailable and therefore titrating the activity of the other pathway.

These possibilities could best be tested under conditions in which Dsh is limiting. Overexpression of Fz causes a dominant gain-of-function PCP phenotype (Fig. 8B), and this phenotype is sensitive to the dose of *dsh* (Krasnow and Adler 1994). To ask whether Wg can cross-activate Dsh activity for PCP signaling, or if it can sequester Dsh, we simultaneously ubiquitously expressed Wg, and asked if the Fz-overexpression phenotype was either enhanced or suppressed. Figure 8C shows that ectopic expression of Wg suppresses the Fz overexpression phenotype, suggesting that activation of Wg signaling may titrate the amount of Dsh available for PCP signaling.

We conducted the reciprocal experiment by asking if ectopic activation of the PCP pathway could interfere with Wg signaling. Because the ligand for PCP signaling is unknown, we overexpressed Fz during embryogenesis,

Figure 8. Reciprocal titration of the PCP and Wg pathways. Wings from wild-type (*A*), *Hs:Fz/+* (*B*), and *Hs:Fz/+; Hs:wg/+* (*C*), each heat-shocked for 1.5 hr at 24 hr AP. Overexpression of Hs:wg alone at this time has no phenotype (not shown). However, it suppresses the Hs:fz overexpression phenotype. Shown are embryos that are wild type (*D*), *U32A/U32A; UASfz/+* (*E*), and *U32A/U32A; UASdsh43-1-B-1/+* (*F*). Whereas overexpression of Dsh produces naked cuticle (*F*), overexpression of Fz leads to lawns of denticles (*E*).

and analyzed the cuticle phenotype. Embryos developed with lawns of denticles and were reminiscent of *wg*-mutant embryos, or those expressing dominant-negative Dsh constructs (Fig. 8E, although note that under heat shock control, Fz expression produces a very weak phenotype reminiscent of Wg overexpression; Tomlinson et al. 1997). Our result suggests that titration can occur in this direction as well. We cannot rule out the possibility that the titration we observed in these experiments results from a promiscuous interaction between Wg and Fz (Bhanot et al. 1996), although this interaction may not occur in vivo (Cadigan et al. 1998). Our observations are equally consistent with the possibility that under these conditions, activity of one pathway titrates the Dsh level available for the other.

Discussion

The role of Dsh in PCP signaling

Through the PCP signal, cells determine their orientation in the plane of the epithelium, and reorganize their cytoskeletons in a polarized array. Although the ligand(s) mediating PCP signaling is not known, the signal must somehow be detected asymmetrically and the response generated asymmetrically by each cell. Recent work suggests that asymmetric distribution or activation of Fz may produce this signal (Adler et al. 1997). Here, we have shown that relocalization of Dsh to the membrane is required for PCP signaling. We speculate that in vivo, asymmetry in PCP signaling results from an asymmetric relocalization of Dsh to the membrane. Dsh could then serve as a marker establishing intrinsic polarity and directing the location of prehair initiation (Wong and Adler 1993). This could occur through polarized cytoskeletal remodelling in a fashion similar, for example, to the mechanism by which *Saccharomyces cerevisiae* orient and grow buds (Chant 1996). A similar mechanism, using Fz and Dsh, may function to orient ommatidia in the developing *Drosophila* eye (Gubb 1993; Strutt et al. 1997; Tomlinson et al. 1997), and may also require activation of the JNK pathway (Strutt et al. 1997; Boutros et al. 1998).

Whereas it is unclear how Dsh is recruited to the membrane, imperfect colocalization suggests that the mechanism does not depend on direct binding of Fz and Dsh. Two mechanisms can be envisioned for its recruitment. First, a signal from Fz may result in a post-translational modification of Dsh, and this modification could then allow it to interact with a target at the membrane. Second, Fz signaling might modify the docking site, which in turn recruits Dsh.

The data presented here do not address directly whether Fz-dependent localization of Dsh to the membrane is signal dependent. Although no exogenous ligand was expressed, it is possible that overexpression of Fz was sufficient to mimic the ligand-bound state. Alternatively, an endogenous ligand might be present that is capable of recognizing and activating Fz. In support of this possibility, a Fz construct lacking the ligand-binding
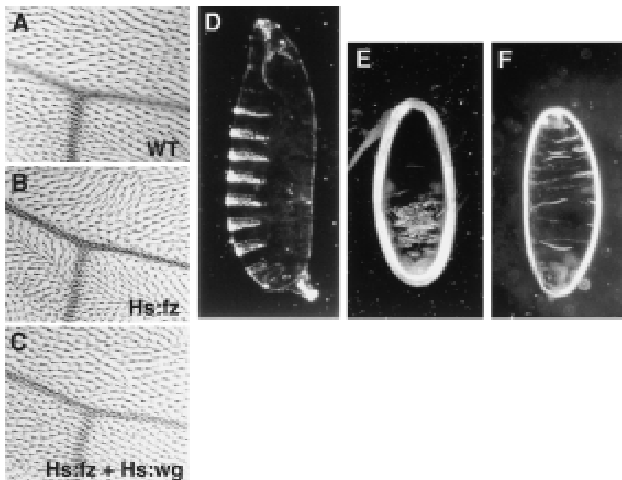
domain (FzΔN) was incapable of inducing membrane localization of Dsh–GFP (not shown).

Functions of Dsh domains in PCP signaling

The functions of Dsh that allow it to participate in discrete signaling mechanisms are not known. The results presented here allow us to draw some inferences concerning the functions of the conserved domains in both pathways, although a more complete understanding awaits identification of the proteins with which these domains interact. In the Fz-mediated pathway, the PDZ domain is not required for translocation to the membrane. However, a protein lacking this domain is dominant negative for PCP signaling, suggesting that the PDZ domain plays a role in PCP signaling, perhaps recruiting another component of the pathway. An interaction with the DIX domain appears to anchor Dsh to cytoplasmic vesicles, but the DIX domain might also participate in an interaction at the membrane.

The DEP domain is both necessary and sufficient for an interaction that brings Dsh to the membrane and filopodia. Expressing just the DEP domain inhibits the function of endogenous Dsh in planar polarity signaling, presumably by blocking this interaction, further suggesting a physiologic importance of this localization. Deletion of the DEP domain produces a dominant-negative protein, indicating that mislocalized Dsh can interfere with PCP signaling, perhaps by nonproductive interaction with another component of the pathway. These results suggest that localization of Dsh is important for PCP signaling, since Dsh molecules that cannot localize properly, or those that block localization of the endogenous Dsh behave as dominant negatives. It will be important to determine the binding partners for the Dsh domains to understand this process in more detail.

DEP domains have been recognized in a group of proteins related by their ability to regulate GTPases: regulator of G-protein signaling (RGS), guanine nucleotide dissociation stimulator (GDS), and GTPase-activating protein (GAP) proteins (Ponting and Bork 1996). Although present in many members of these groups, other domains are responsible for directly interacting with and regulating the GTPases. DEP domains are therefore likely involved in a different level of regulation. Our data indicate that the Dsh DEP domain is important for localization of Dsh during PCP signaling. egl10, an RGS protein in *Caenorhabditis elegans,* also displays a restricted subcellular localization that depends on its DEP domain (Koelle and Horvitz 1996). DEP domains may therefore serve to localize proteins to specific compartments in the cell.

The involvement of other DEP domain proteins in regulation of GTPases leads us to consider the possibility that Dsh also plays such a role. As noted previously, the small GTPases RhoA and Rac1 appear to be important for PCP signaling (Eaton et al. 1995, 1996; Strutt et al. 1997a). It is also possible that heterotrimeric G proteins play a role. PCP signaling may work by a mechanism homologous to the Wnt5a signaling pathway in *Xenopus,* in which a G-protein mechanism has been implicated (Slusarski et al. 1997a). It may also be homologous to a Wnt mediated polarity signal in *C. elegans* embryos (Rocheleau et al. 1997; Thorpe et al. 1997), which also requires a G protein (Zwaal et al. 1996). It will be important to determine what interactions might exist between Dsh and GTPases in PCP signaling.

Functions of Dsh domains in Wg signaling

It is more difficult to make specific inferences regarding the functions of these domains in Wg signaling. The DEP domain is not essential for Wg signaling, as Dsh(Δ*EP+) retains function. We note that the PDZ domain is dispensable for Wg signaling when Dsh is expressed at high levels, as was done in the embryo-injection rescue assay, but may have an important function when the protein is expressed at wild-type levels (Yanagawa et al. 1995; see below). One possibility is that the PDZ is used to localize Dsh but that overexpression allows sufficient levels of Dsh(ΔbPDZ) to reach the appropriate location. Overexpression of the PDZ domain could therefore interfere with this localization, resulting in dominant interfering activity.

In a cell-culture assay for Wg signaling, Dsh induced accumulation of Arm; however, a Dsh construct lacking the PDZ domain failed to do so (Yanagawa et al. 1995). In addition, a *Xenopus* Dsh lacking the PDZ functioned as a dominant negative (Sokol et al. 1996). Although we cannot explain fully the apparent discrepancy between these results and ours, in both these experiments, the truncated proteins were expressed in a wild-type background, whereas in the experiment we have presented, rescue was assessed in a *dsh* null mutant background. When Dsh(ΔbPDZ) mRNA was injected into wild-type embryos, we observed a modest degree of denticle fusions that might be interpreted as dominant-negative activity (data not shown). We conclude that Dsh(ΔbPDZ) can rescue null mutants, but may function as a weak dominant negative in the presence of wild-type Dsh.

Differential activation of Dsh

We have presented five lines of evidence indicating that Dsh transduces two distinct signals, the Wg and PCP signals, as a result of its differential activation by the two pathways: (1) Dsh is recruited differentially to the membrane by the putative receptors for the two pathways; (2) the *dsh[1]* allele selectively affects PCP signaling, and encodes a structural alteration in a domain required for translocation to the membrane; (3) domains in Dsh are active differentially in PCP and Wg signaling; (4) downstream components of the two pathways are not shared; and (5) Dsh function in one pathway can be titrated by activation of the other pathway.

In *Xenopus* animal caps, Dsh is recruited to the membrane or cell cortex in response to Fz, and is also induced to associate with filopodia. We propose that a similar localization is likely to occur during PCP signaling, although it may be only a fraction of the Dsh that localizes in vivo, and the relocalization may be restricted in a

signal-dependent fashion to a small portion of the cell (see below). The small size of the cells would make this very difficult to detect.

In contrast, DFz2, the putative Wg receptor, appears not to induce relocalization of Dsh, regardless of the presence of ligand. One of two possible conclusions may be drawn from these obervations. The results might indicate that Dsh is activated differently by the PCP and Wg pathways; this conclusion would be consistent with the other data presented here. In support of this model, *Xenopus* Wnt8 (XWnt8), which, like Wg, induces axis duplication by activating the Wnt pathway, fails to induce membrane localization of *Xenopus* Dsh (Yang-Snyder et al. 1996). Therefore, functional activation of the Wnt (Wg) pathway by either Wg or XWnt8, fails to localize Dsh to the membrane.

An alternative explanation for the failure of DFz2 to recruit Dsh to the membrane is that multiple receptors are required to transduce a complete response to Wg, or that DFz2 does not function as the Wg receptor in vivo, despite its ability to transduce the Wg signal in cultured cells (Bhanot et al. 1996). Consistent with the former possibility, it has been argued that distinct domains in Wg activate different subsets of the Wg response (Bejsovec and Wieschaus 1995; Hays et al. 1997). The receptor Notch has also been proposed to function as a Wg receptor (Couso and Martinez-Arias 1994). We found that Notch also failed to recruit Dsh to the membrane in the *Xenopus* animal cap assay (not shown); we found previously that colocalization in S2 cells required capping by Dl-expressing cells (Axelrod et al. 1996). Additional analyses of Notch, DFz2, and other candidate receptors, will be required to asses their possible roles in Wg signaling.

We cannot rule out the possibility that in *Drosophila*, some polarity information is transduced along with cell fate information by Wg signaling (Thiesen et al. 1994; Tomlinson et al. 1997), or that Fz is required for mediation of Wg signaling in addition to its role in PCP signaling (Bhanot et al. 1996). In other words, the specificity of these pathways may be partial rather than absolute. In this context, it is interesting to note that in cultured *Drosophila* cells, a small fraction of Dsh was found in the membrane fraction of Wg treated cells (Yanagawa et al. 1995).

Divergence of Wnt pathways in other organisms

Evidence for a divergence of Fz-mediated signaling pathways can also be found in other organisms. In *C. elegans,* genes encoding members of a Wnt signaling pathway are required for a descendent of the EMS blastomere to adopt the E fate (Rocheleau et al. 1997; Thorpe et al. 1997). In addition, the rotation of a number of mitotic spindles, including that of the EMS cell, produces a polarized cell division, and depends on Wnt activity [and on G-protein function (Zwaal et al. 1996)]. Induction of the E cell fate was found to require the homologs of Wnt, Fz, Arm, and Pan, but only the Wnt and Fz homologs were required for polarization of the EMS and other cells. These results

suggest that there is a divergence in the signaling pathway downstream of Fz but upstream of Arm.

Additional evidence of distinct functions for Dsh comes from observations in mice and chicks. Although little data concerning their function exists, it has been observed that in PC12 cells, two of the three murine Dsh homologs, Dvl-1 and Dvl-2, respond to Wnt-1 by adopting different subcellular localizations: Dvl-1 localizes to the membrane, whereas Dvl-2 localizes to the cytoskeleton (Steitz et al. 1996). It is tempting to speculate that each of these Dsh homologs is involved differentially in transducing polarity and cell fate or other distinct signals. Similarly, in the chick limb bud, Wnt3a and Wnt7a induce distinct responses in the mesoderm that are β-catenin dependent and independent, respectively (Kengaku et al. 1998).

Studies of Wnt signaling in *Xenopus* provide evidence of divergent pathways as well. *Xenopus* Wnt1 (XWnt1) induces axis duplication by activating a pathway similar to that used by Wg. In contrast, *Xenopus* Wnt5a (XWnt5a) fails to induce axis duplication, but rather alters morphogenetic movements during gastrulation (Moon et al. 1993), perhaps by activating a G-protein coupled pathway resulting in decreased cell adhesion and increased intracellular Ca^{2+} levels (Slusarski et al. 1997a,b). A difference in the ability of rFz1 and rFz2 to activate these pathways has also been observed (Yang-Snyder et al. 1996; Slusarski et al. 1997a; J.D. Brown and R.T. Moon, unpubl.). Of note, XWnt5a blocks the axis-duplicating activity of XWnt1, but not that of downstream components of the XWnt1 pathway, β-catenin or kinase dead-Gsk-3 (Torres et al. 1996). This is reminiscent of the ability of Wg to block signal transduction in the PCP pathway and vice versa, and might occur through a similar mechanism of titrating Dsh. In contrast, a heterologous Fz, human Fz5 (hFz5), can serve as an ''adaptor'', allowing XWnt5a to induce axis duplication (He et al. 1997). hFz5 appears to bind XWnt5a, but has the specificity to activate the Wg-like axis duplicating pathway. Therefore, Fz pathways in *Xenopus* have differing specificities, and may functionally titrate each other. The specificity with which the pathways are activated appears to be a function of the Fz protein(s) recognized by each Wnt.

Conclusions

Few well understood examples of how extracellular signals regulate the cytoskeleton exist. Dsh links the activity of the Fz family of receptors to control of the cytoskeleton, and we propose that it does so by a mechanism distinct from its function in Wg signal transduction. Since it lacks recognizable sequences suggesting an enzymatic activity, Dsh may serve as a scaffolding or adapter protein. PDZ domains are found in such proteins, but the functions of DEP and DIX domains are yet to be defined. Elucidation of Dsh function in PCP and Wg signaling may shed light on the functions of these motifs, as well as enlighten our understanding of how

Dsh may contribute to the specificity of Fz-mediated signaling.

Materials and methods

Overexpression and generation of mutant clones

zw3, arm, and *arm^S10* (Pai et al. 1997), *wg* (Noordermeer et al. 1992), *fz* (Krasnow and Adler 1994; Adler et al. 1997), *fzΔN, dsh* [two insertions were used: T8 (Axelrod et al. 1996), and T15 (on the third chromosome)], and the *dsh* deletion constructs were overexpressed using either the heat shock promoter in pCaSpeR–hs (Thummel et al. 1988; Thummel and Pirotta 1992), or by using pUAST (Brand and Perrimon 1993) crossed with hs-GAL4 (Brand et al. 1994) or the maternally expressed U32A:GAL4 (*matα*–GAL4–VP16, containing amino acids 1–144 of GAL4 fused to the transcriptional activator domain of VP16, and expressed from the α4–tubulin promoter, was a gift of D. St Johnston, Wellcome/CRC Institute, Cambridge, UK). Transformants were obtained as described (Spradling 1986; Robertson et al. 1988). Heat shocks at 37°C were applied for the indicated times and durations. Where appropriate, overexpression was done in *dsh^1*/*Y* males, or in the presence of *UAS:dsh* or *Hs:dsh.*

Clones of homozygous *zw3* or *arm*-mutant cells were generated using the FLP technique (Chou and Perrimon 1992). Somatic clones were produced by crossing *FM7/y arm^H8.6 f^36a FRT^9-2* or *FM7/Df(1) sc^B57 sgg^D127 f^36a FRT^19A* females to *ovo^D2 FRT^9-2; FLP^38/FLP^38* or *FRT^19A/Y; MKRS, FLP^M42/+* males, and heat-shocking at 37°C for 2 hr during third instar. *FRT^9-2, FLP^38, MKRS, FLPM^42,* and *FRT^19A* are as described by Chou and Perrimon (1992), Siegfried et al. (1994), and Xu and Rubin (1993), respectively. The *ovo^D2* mutation produces dominant female sterility and has no effect on development of somatic tissues. Flies bearing *arm^H8.6* clones were raised at 29°C because of the temperature sensitivity of the allele (Klingensmith et al. 1989).

All wings were mounted in Euparal (Asco Laboratories, Manchester, UK) for examination and photography.

dsh^1 cloning and sequencing

Total genomic DNA from *dsh^1* males was isolated, cut with *Sal*I and *Xba*I, size fractionated, and cloned into pBSK (Stratagene). Clones were screened by hybridization with *dsh* cDNA. Positives were sequenced. The *dsh^1* mutation was recreated in a wild-type *dsh* genomic fragment, cloned into pCaSpeR (Thummel et al. 1988), and transformants generated (Spradling 1986; Robertson et al. 1988). Flies carrying the introduced gene,

in up to three copies, in a *dsh^v26* null mutant background were viable, and had the *dsh^1* phenotype.

Xenopus animal cap explants and confocal microscopy

Capped RNAs encoding each cDNA construct were synthesized with the mMessage Machine kit (Ambion, Austin, TX) from linearized DNA templates. The indicated RNAs (250–500 pg) were injected into the animal pole of two blastomeres at the four-cell stage and injected embryos were cultured to stage 9. Animal caps dissected from injected embryos were fixed in 4% paraformaldehyde, 10 mM HEPES (pH 7.6), 100 mM KCl, 3 mM MgCl$_2$, 2 mM EGTA, and 150 mM sucrose for 1–2 hr at room temperature. For colocalization experiments, fixed animal caps were dehydrated in MeOH after which they were immunostained in PBST + 10% goat serum with either anti-DFz1 antibodies (1:1000 dilution; Park et al. 1994) or anti-human c-*myc* 9E10 monoclonal antibodies (1:25 dilution). Cy3-conjugated secondary antibodies (1:250 dilution; Jackson ImmunoResearch, West Grove, PA) were used to detect the localization of ectopic Fzs. The distribution of F-actin in animal cap cells was visualized with Texas Red-conjugated phalloidin (Molecular Probes, Eugene, OR). Animal caps were mounted in Vectashield for microscopy (Vector Laboratories, Burlingame, CA).

The localization of ectopic GFP-tagged Dsh and Fz proteins in *Xenopus* animal cap cells was determined by laser scanning confocal microscopy. Confocal microscopy was performed using a Bio-Rad MRC600 scan head (Bio-Rad Labs, Hercules, CA) and a Nikon Optiphot-2 microscope (Nikon, Melville, NY). All images were collected with a 60 × 1.4 NA PlanApo objective. Multilabeled samples were scanned sequentially using appropriate filter blocks to ensure that there was no bleedthrough of fluorescence between channels. Digital images were processed using Adobe Photoshop software (San Jose, CA).

RNA injection rescue assay

Capped RNAs for Dsh and the deletion constructs were synthesized in vitro and injected into *svb dsh* germ-line clone embryos or their paternally rescued siblings, and cuticles prepared and examined as described previously (Klingensmith et al. 1996).

Expression constructs

Dsh deletion constructs span the following coordinates (expressed as amino acid position, according to Klingensmith et al. 1994): Dsh (1–623); Dsh(ΔDIX) (1–41 + 152–623); Dsh(ΔbPDZ)

Table 1. *Behavior of Dsh deletion constructs in the structure/function assays*

Construct	PCP assay				Wg assay		
	in wild type	in *dsh^1*	in *Hs:dsh*	summary	in wild type	in *dsh^V26*	summary
Dsh	+++[a]	+++ (rescue)		+++[b]	+/–[c]	+++ (hatch)	+++[b]
Dsh(ΔDIX)	+/–			?	–	++	+
Dsh(ΔbPDZ)	++	–		DN	+/–DN[d]	+++ (hatch)	+++(+/–DN)
Dsh(ΔDEP+)	++	–		DN	–	–	–
Dsh(Δ*EP+)					–	++	++
Dsh(DIX)	–			–	DN	–	DN
Dsh(bPDZ)	–			–	DN	–	DN
Dsh(DEP+)	++	–	rescue	DN	–	–	–

[a]Dominant overexpression phenotype.
[b]Functional.
[c]Slight naked phenotype consistent with Dsh or Wg overexpression.
[d]Slight DN phenotype.

(1–151 + 334-623); Dsh(ΔDEP+) (1–394); Dsh(Δ*EP+) (1–423); Dsh(DIX) (1–101 + 582–623); Dsh(bPDZ) (1–41 + 152–394); Dsh-(DEP+) (1–41 + 334–623); Dsh(ΔDEP) (1–394 + 481–623). GFP(F64L,S65T) fusions were made after aa 621. FzΔN had amino acid 29–242 deleted. Fz lacking the carboxyl terminus had the last six amino acids deleted and substituted with PSRASRTIVSRIT. Details of these constructions are available on request. Myc–DFz2 had six copies of the myc 9E10 epitope inserted in the *Apa*I site in the extracellular domain. The behavior of these constructs in the structure/function assays are shown in Table 1.

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