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Interactions between Wingless and DFz2 during *Drosophila* wing development

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SUMMARY

***Drosophila* Wingless (Wg) is a secreted signaling protein of the Wnt family. Mutations in the *wg* gene disrupt the patterning of embryonic segments and their adult derivatives. Wg protein has been shown in cell culture to functionally interact with DFz2, a receptor that is structurally related to the tissue polarity protein Frizzled (Fz). However, it has not been determined if DFz2 functions in the Wg signaling pathway during fly development. Here we demonstrate that overexpression of DFz2 increases Wg-dependent signaling to induce ectopic margin bristle formation in developing *Drosophila* wings. Overexpression of a truncated form of DFz2 acts in a dominant-negative manner to block Wg signaling at the wing margin, and this block is rescued by co-expression of full-length DFz2 but not full-length Fz. Our results suggest that DFz2 and not Fz acts in the Wg signaling pathway for wing margin development. However, a truncated form of Fz also blocks Wg signaling in embryo and wing margin development, and the truncated form of DFz2 affects ommatidial polarity during eye development. These observations suggest that a single dominant-negative form of Fz or DFz2 can block more than one type of Wnt signaling pathway and imply that truncated proteins of the Fz family lose some aspect of signaling specificity.**

Key words: *Drosophila*, Frizzled, DFz2, Wingless, Bristle formation, Cell signalling

INTRODUCTION

Members of the Wnt family of secreted proteins play important roles in animal development (Nusse and Varmus, 1992). The *Drosophila* Wnt-1 ortholog, Wingless (Wg), functions as an inductive signal during both embryonic and imaginal development of the fruit fly. During embryogenesis, Wg is required to establish the anterior/posterior (A/P) polarity of each body segment (Bejsovec and Martinez-Arias, 1991). Wg is also required for the patterning of the adult eyes, legs and wings (Couso et al., 1993; Struhl and Basler, 1993; Couso et al., 1994; Heberlein et al., 1998). In the wing imaginal disc, Wg is required at different steps of development. Wg is first expressed in the ventral compartment where it is required for correct dorsal/ventral (D/V) patterning (Williams et al., 1993; Couso et al., 1994). Subsequently, Wg is expressed in a stripe of cells at the presumptive wing margin where it induces the differentiation of wing margin structures including sensory bristles (Phillips and Whittle, 1993; Couso et al., 1994). In the developing eye disc, Wg activity is required to establish the eye D/V midline and presumptive equator (Heberlein et al., 1998).

Based primarily on genetic analysis, it has been proposed that the Wg signal is transduced through a common intracellular pathway within responding cells in each of these tissues (Heslip et al., 1997). When cells receive a Wg signal, the cytoplasmic Dishevelled (Dsh) protein inhibits the activity of a serine-threonine kinase, Zeste White-3 (ZW3) (Siegfried et al., 1990; Noordermeer et al., 1994). In the absence of a Wg signal, non-repressed ZW3 produces rapid turnover of a β-catenin encoded by the *armadillo* (*arm*) gene (Peifer et al., 1994). When ZW3 is repressed by Wg and Dsh, Arm protein is stabilized and transported to the nucleus where it interacts with TCF to activate gene transcription (Brunner et al., 1997; Riese et al., 1997; van de Wetering et al., 1997). The Wg signal transduction pathway is strikingly similar to the vertebrate Wnt pathway in both the identities and order of signaling molecules (Parr and McMahon, 1994).

Dsh and ZW3 are also required to impart polarity to imaginal cells. In many epidermal adult tissues of *Drosophila*, establishment of hair and bristle polarity requires the activity of Dsh (Gubb and Garcia-Bellido, 1982). Moreover, Dsh and ZW3 are required to coordinate ommatidial polarity in the retina of the compound eye (Theisen et al., 1994; Zheng et al., 1995; Tomlinson et al., 1997). Epidermal and retinal polarity also requires the activity of Frizzled (Fz) (Gubb and Garcia-Bellido, 1982; Vinson and Adler, 1987; Zheng et al., 1995). Fz is a seven-transmembrane protein localized to the surface of cells within developing imaginal discs (Park et al., 1994). Wg protein can physically associate with S2 cells in culture expressing either Fz or DFz2, a *Drosophila* protein closely related to Fz, suggesting that they may function as Wg

receptors (Bhanot et al., 1996). Moreover, DFz2 promotes stabilization of Arm in response to Wg. In *Xenopus* embryos, Rat Fz1 mediates XWnt-8 signaling and Human Fz5 mediates XWnt-5A signaling (Yang-Snyder et al., 1996; He et al., 1997). Together, these data suggest that the Fz family of proteins are Wnt receptors.

Despite the fact that both Fz and DFz2 interact with Wg in cell culture (Bhanot et al., 1996), it has not been shown that they act as Wg receptors during fly development. To address this question, we present genetic evidence suggesting that DFz2 and not Fz acts in the Wg signal transduction pathway during wing development. This evidence is based on misexpression of full-length and dominant-negative receptors in the developing wing. We present further evidence to suggest that unknown specificity factors shield each receptor from activating both polarity and differentiation responses in cells.

MATERIALS AND METHODS

Construction of transgenes and generation of transgenic flies

The Fz cDNA sequence from nucleotides 374 to 2360 (Vinson et al., 1989), which contains the entire open reading frame, was cloned into pUAST (Brand and Perrimon, 1993) to generate pUAS-Fz. pUAS-FzN was prepared by cloning the Fz cDNA sequence from nucleotides 589 to 1413 into pUAST. This includes coding sequence for the N-terminal 272 amino acids which encompasses the extracellular domain, the first transmembrane domain and the first two residues of the first intracellular loop. This was put in frame with a heterologous peptide RWL followed by a stop codon. UAS-Fz and UAS-FzN also differed from each other in that the latter contained 9 nucleotides of 5′-UTR and the former contained 224 nucleotides of 5′-UTR. However, this difference in the amount of 5′-UTR does not likely affect the level of gene expression of either transgene. We fused the Fz coding region and either 9 or 224 nucleotides of 5′-UTR to the heat shock promoter in pCasper-hs. Transformant lines carrying either of these transgenes exhibited equally severe polarity phenotypes when flies were heat shocked (data not shown).

To generate the DFz2 constructs, a short region of DFz2 was amplified from an eye disc cDNA library using PCR and was subsequently used as a probe to screen the eye disc cDNA library. A DFz2 cDNA encompassing the entire open reading frame was then subcloned into the *Eco*RI site of pUAST to make pUAS-DFz2. pUAS-DFz2N was made by substituting pUAS-DFz2 coding sequence downstream of a *Bam*HI site at nucleotide 174 (Bhanot et al., 1996) with coding sequence from DFz2 (nucleotides 174-1030) plus sequence encoding R-W-L-Stop. This generated a fusion of extracellular and first transmembrane domains with an intracellular DTRWL at the carboxy terminus, analogous to the mutation made in pUAS-FzN. Multiple independent lines of transgenic flies were generated by P-element mediated transformation.

Fly stocks

Gal4 lines and UAS-*lacZ* have been described (Brand and Perrimon, 1993; Speicher et al., 1994; Staehling-Hampton et al., 1994). *wg-lacZ* (Kassis et al., 1992), *neu-lacZ*(Phillips et al., 1990) and *vg-lacZ* (Williams et al., 1994) have been characterized. The mutations in Wg pathway were previously characterized as strong alleles: dsh^{v26} (Theisen et al., 1994), arm^{XK22} (Peifer and Wieschaus, 1990), vg^{1} (Williams et al., 1993), $zw3^{m11}$ (Blair, 1994), and wg^{CX4} (Baker, 1987).

Examination of embryonic cuticles and adult structures

Embryonic cuticles were prepared as described by Zhu and Kuziora (1996) and viewed with dark-field microscopy. Adult wings were dissected in 95% ethanol and mounted in 50% lactic acid in ethanol.

Histochemistry

Dissection of imaginal discs and X-Gal staining were done as described by Zheng et al. (1995). The antibody staining with monoclonal anti-Achaete antibody, kindly provided by Dr Carroll, was performed as described by Skeath and Carroll (1991).

RESULTS

Overexpression of Dsh activates the Wg signaling pathway in vivo and in vitro (Yanagawa et al., 1995; Axelrod et al., 1996). If DFz2 or Fz is the Wg receptor, we reasoned that overexpression of these proteins might also activate the Wg signaling pathway. To test this hypothesis, we overexpressed either Dz2 or Fz in the developing wing blade. The Gal4/UAS system was used to drive ectopic DFz2 expression (Brand and Perrimon, 1993). The 69B-Gal4 driver activates UAS-responsive gene expression throughout the wing blade region of a third-instar wing imaginal disc, as demonstrated by UAS-*lacZ* expression (Brand and Perrimon, 1993; Fig. 1M,N). Animals expressing the UAS-*DFz2* transgene under 69B-Gal4 control formed ectopic wing margin bristles in the interior of the wing (Fig. 1D). Alongside the anterior margin, supernumerary stout mechanosensory bristles were often observed one or more cell diameters away from the normal stout bristle row (compare Fig. 1E with wild-type in Fig. 1B). Within the wing, supernumerary chemosensory and slender mechanosensory bristles were detected (Fig. 1E,F). The bristle types (stout, slender and chemosensory) were located on the appropriate dorsal or ventral surface of the wing, and were restricted to the anterior region of the wing. Alongside the posterior wing margin, supernumerary non-sensory bristles were observed several cell diameters distance from the margin where these bristles are normally located (Fig. 1G). The formation of supernumerary bristles by DFz2 did not appear to result from a disturbance in lateral inhibition between neighboring bristle precursor cells. Contrary to what is typically seen when lateral inhibition is perturbed, there was no overall change in margin bristle density. The spacing between bristles within the three regular rows of anterior bristles and within the single row of posterior bristles of 69B-Gal4/UAS-*DFz2* wings was indistinguishable from that of wild type (compare Fig. 1B-C with Fig. 1E-G).

These phenotypes are similar to supernumerary bristle phenotypes observed when Wg or Dsh are ectopically expressed during the time of endogenous bristle induction (Axelrod et al., 1996). Indeed, Wg is believed to be the inductive signal controlling margin bristle development. Several observations suggest that formation of ectopic bristles by UAS-*DFz2* depends on the level of Wg signaling. First, the ectopic bristle response is completely suppressed in 69B-Gal4/UAS-*DFz2* flies heterozygous for a null *wg* allele (Fig. 1H). Second, ectopic bristles are more numerous near the margin, where endogenous Wg is most highly expressed (Fig. 1E). Third, the type of ectopic bristle formed correlates with its distance from the margin; supernumerary bristles close to the margin are invariably of the stout type, and bristles distant from the margin are of the slender or chemosensory types. These observations suggest that DFz2

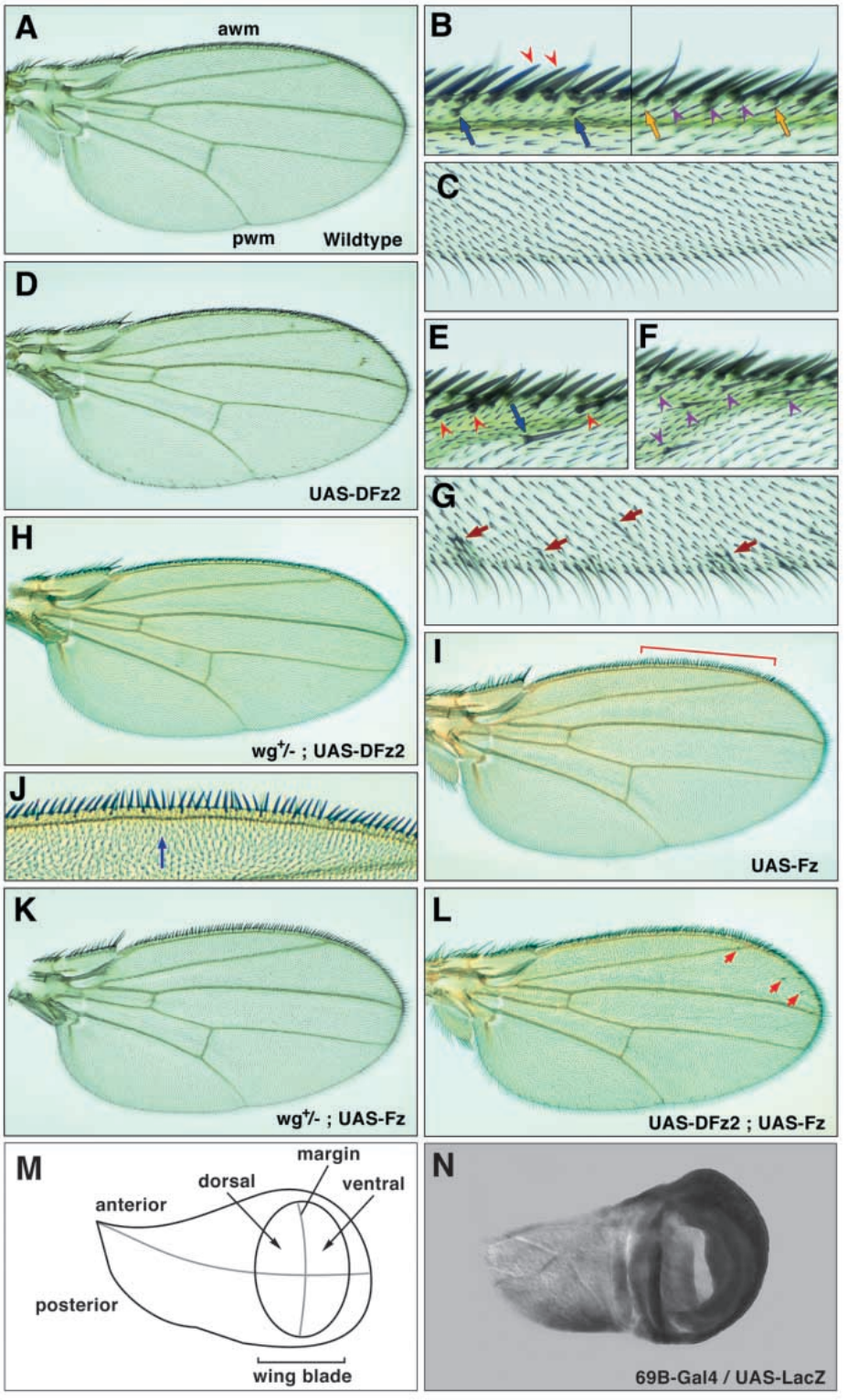

Fig. 1. Overexpression of DFz2 and Fz perturb margin bristle induction and polarity, respectively. (A) A wild-type wing. The wing margin is composed of two types of bristle elements. Along the anterior wing margin (awm), three rows of chemosensory and mechanosensory bristles are found. Along the posterior wing margin (pwm), non-innervated bristles are patterned in two rows. (B) Detail of the triple row at the anterior margin at two different planes of focus, showing the two dorsal bristle rows in the left panel and the single ventral row in the right panel. Dorsally, there is a row of densely packed stout mechanosensory bristles (red arrowheads) closest to the edge. This is followed by a row of hairs and then a row of chemosensory bristles (blue arrows), each interspersed by four hairs. Ventrally, there is a row of bristles containing a sequence of one recurved chemosensory bristle (yellow arrow) between every three to four slender mechanosensory bristles (purple arrowheads). (C) Detail of the bristle rows at the posterior margin. Two rows of alternating curved bristles are seen. No bristles are innervated. (D) A wing from a 69B-Gal4/UAS-*DFz2* fly. Although most wing structures develop normally, ectopic margin bristles are induced close to the wing margin and are seen as dark structures in the wing interior. (E) At the dorsoanterior wing margin of a 69B-Gal4/UAS-*DFz2* wing, supernumerary stout mechanosensory bristles (red arrowheads) are induced one or two rows away from the normal central row. Supernumerary chemosensory bristles (blue arrow) are induced several rows away from the margin. (F) At the ventral-anterior margin of a 69B-Gal4/UAS-*DFz2* wing, supernumerary slender mechanosensory bristles (purple arrowheads) are located one or more rows from the margin. (G) Supernumerary posterior margin bristles (red arrows) can be found in a 69B-Gal4/UAS-*DFz2* wing. (H) The supernumerary bristle phenotype is suppressed in a 69B-Gal4/UAS-*DFz2* animal that is also heterozygous for a loss-of-function *wg* ^{CX4} mutation. (I) A wing from a 69B-Gal4/UAS-*Fz* fly. Margin structures are properly patterned but polarity of hairs and margin bristles is perturbed. Margin bristles under the red bracket are pointing straight out or proximally rather than pointing in a distal direction as seen in wild type. (J) Detail of anterior wing margin of Gal4/UAS-*Fz* fly showing altered polarity of margin bristles and interior wing hairs (blue arrow). (K) The polarity phenotype is unchanged in a 69B-Gal4/UAS-*Fz* animal that is also heterozygous for a loss-of-function *wg* ^{CX4} mutation. (L) A wing from an animal that expresses both UAS-*DFz2* and UAS-*Fz* under control of the 69B-Gal4 driver. Supernumerary margin bristles (red arrows) and perturbed bristle and hair polarity are observed. (M) Diagram of a late third instar wing imaginal disc. Dorsal and ventral regions of the prospective wing blade are separated by the prospective wing margin, indicated by a gray line. The boundary between the anterior and posterior compartments is also indicated by a gray line. In the adult wing blade, the ventral region is folded under the dorsal region to form a two-layered wing blade. The point of intersection between the A/P and D/V boundaries forms the distal tip of the adult wing. (N) Pattern of 69B-Gal4 expression in a late third instar wing disc is revealed by detection of β-galactosidase activity in a 69B-Gal4/UAS-*lacZ*. animal. Expression is nearly ubiquitous within the prospective wing blade though it is stronger in the ventral region than in the dorsal region.

overexpression potentiates endogenous Wg signaling from the margin.

In contrast, no ectopic margin bristles were present in animals expressing UAS-*Fz* under 69B-Gal4 control. However, the polarity of margin bristles was abnormal, and wing hair polarity was deranged throughout the plane of the wing blade (Fig. 1I,J). Similar results were observed by Krasnow and Adler (1994) using a hs-Fz transgene. Reduction of Wg activity had no discernible effect on the strength of the abnormal polarity phenotype (Fig. 1K). The differences between overexpressed DFz2 and Fz with respect to their wing phenotypes and their sensitivities to Wg dosage suggest that the two proteins mediate separate signaling pathways. Consistent with this conclusion, overexpression of both DFz2 and Fz in the same wing produces a phenotype that is simply the sum of the two separate wing phenotypes (Fig. 1L). We propose that a similar independence exists between the Fz and DFz2 pathways during wild-type wing development.

Because Fz also establishes ommatidial polarity in the compound eye, we tested whether overexpressing Fz and DFz2 affects eye polarity. Accordingly, Fz or DFz2 were overproduced in the *sevenless* expression domain of differentiating photoreceptor and cone cells using a Sev-Gal4 driver. In wild-type eyes, ommatidia occur in two chiral patterns, one form is exclusively located in the dorsal half of the eye and its mirror form is exclusively located in the ventral half of the eye (Fig. 2A,B). As observed by Strutt et al (1997) and Tomlinson et al (1997), when Fz was overproduced in the *sevenless* expression domain, ommatidia were misoriented and the two chiral forms were not strictly segregated to either half of the eye (Fig. 2C,D). This phenotype is similar to the phenotype of *fz* loss-of-function mutants (Zheng et al., 1995), suggesting that the level of Fz activity in cells is critical for the coordinate patterning of ommatidia. In contrast, overexpression of DFz2 with Sev-Gal4 had no effect on ommatidial polarity (Fig. 2E,F). This result is consistent with the normal epidermal hair and bristle polarity observed in 69B-Gal4/UAS-*DFz2* animals.

Various receptor studies have shown that dominant-negative inhibition of a receptor can be achieved by coexpressing a structural truncation of the receptor. We utilized this approach by generating truncated forms of Fz and DFz2 in which only the extracellular domain and first transmembrane domain was

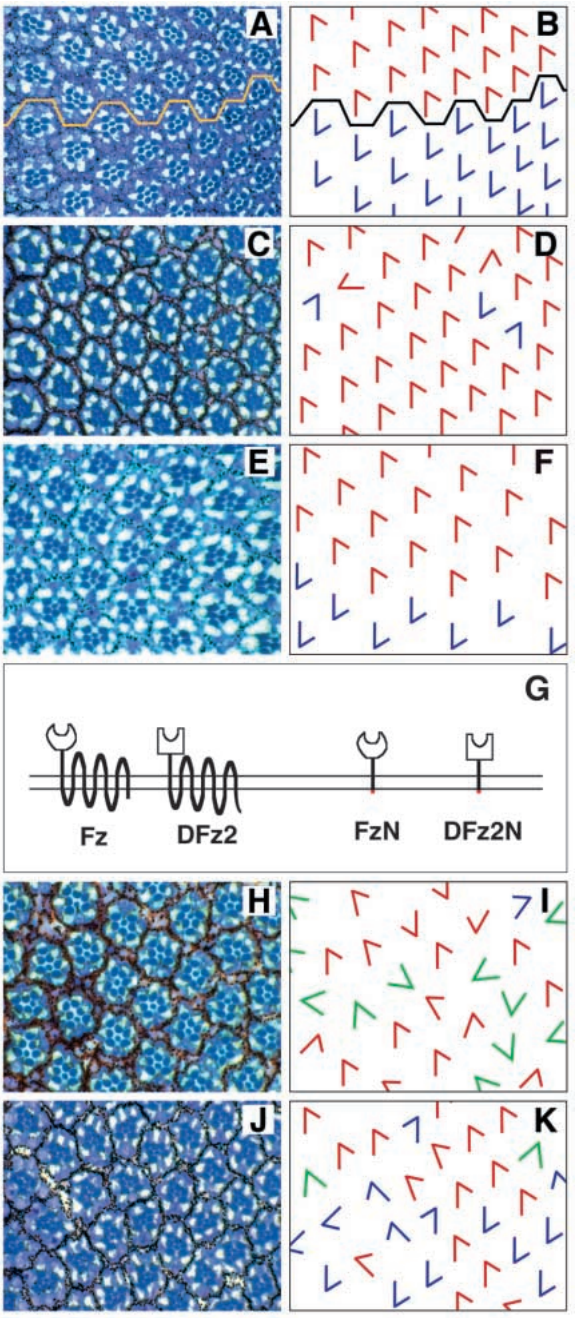

Fig. 2. Ommatidial polarity in adult eyes is affected by Fz. (A,C,E,H,J) Tangential sections through eyes with anterior to the left and dorsal to the top. (B,D,F,I,K) Corresponding interpretations of ommatidial polarity from tangential sections. Ommatidia are represented by one-sided arrows; the long side is aligned with the rhabdomeres (darkly staining circular organelles) of photoreceptors R1, R2, and R3, and the short side is aligned with the rhabdomeres of photoreceptors R3, R4, and R5. Ommatidial polarity is color-coded: red and blue identify chiral forms that reflect across the equator; green identify forms with bilateral symmetry and therefore no chirality. (A,B) Wild-type eye. The equator (outlined with orange or black lines) is a line of mirror image symmetry generated by reflection of the two opposite chiral forms of ommatidia. (C,D) Eye from Sev-Gal4/UAS-*Fz* fly. The two chiral forms are intermixed and are no longer strictly segregated to opposite sides of an equator. Ommatidia are not all uniformly oriented in parallel or anti-parallel directions but are oriented randomly. These phenotypes are similar to loss-of-function alleles of *fz*. (E,F) Eye from Sev-Gal4/UAS-*DFz2* fly shows a normal organization of ommatidial polarity. (G) Schematic representation of the proteins used in this study. DFz2 and Fz have a similar structure with seven transmembrane domains and an extracellular domain containing a conserved cysteine-rich region. FzN is a truncated form of Fz which retains only the extracellular domain and the first transmembrane domain (residues 1-272) fused to a heterologous cytoplasmic tail (DSRWL) marked by a red box. DFz2N is a truncated form of DFz2 which retains only the extracellular domain and the first transmembrane domain (residues 1-343) fused to a heterologous cytoplasmic tail (DTRWL) marked by a red box. (H,I) Eye from a Sev-Gal4/UAS-*FzN* fly. The two chiral forms are intermixed and many symmetric non-chiral ommatidia are observed. Ommatidia are not oriented parallel or anti-parallel to each other but are oriented in many different directions. (J,K) Eye from a Sev-Gal4/UAS-*DFz2N* fly. The phenotype is similar to that observed in Sev-Gal4/UAS-*FzN* eyes.

present (Fig. 2G). The genes encoding the two truncated proteins were placed under control of a UAS enhancer, and UAS-*FzN* or UAS-*DFz2N* expression was then directed in eyes by the Sev-Gal4 driver. Overexpression of either DFz2N or FzN resulted in abnormal ommatidial polarity (Fig. 2H-K). The phenotypes resembled the disordered patterning seen in the eyes of *fz* loss-of-function mutants. In contrast to full-length DFz2, overexpression of DFz2N disrupted polarity patterning in the eye.

To elucidate the effects of FzN and DFz2N on wing margin development, they were overexpressed in wing imaginal discs using the 69B-Gal4 driver line. Expression of DFz2N resulted in significant wing margin and bristle loss, and reduction in wing size (Fig. 3A). Expression of FzN resulted in partial loss of posterior wing margin and partial loss of anterior margin bristles (Fig. 3B). These phenotypes are similar to those seen when Wg activity is genetically reduced in the wing (Couso et al., 1994). The wing margin develops from a D/V boundary that subdivides the wing disc (Diaz-Benjumea and Cohen, 1993; Williams et al., 1993, 1994). Early loss of Wg activity

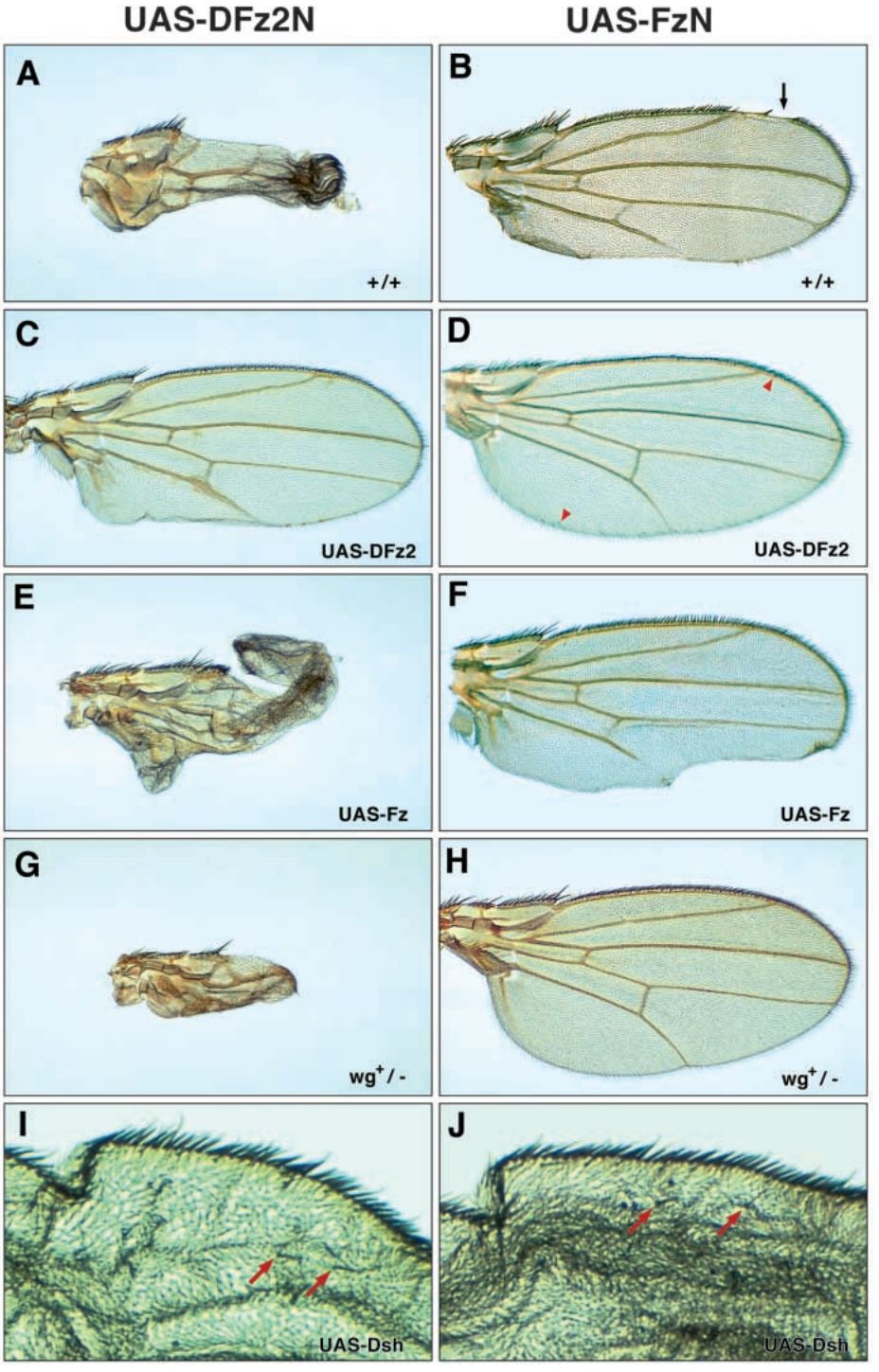

Fig. 3. DFz2N and FzN disrupt wing patterning. (A,C,E,G,I) Wings from animals with UAS-*DFz2N* under 69B-Gal4 driver control. (B,D,F,H,J) Wings from animals with UAS-*FzN* under 69B-Gal4 control. (A) Major wing margin structure loss is observed in a 69B-Gal4/UAS-*DFz2N* wing so that only the most interior structures are retained. (B) Wing margin loss is less severe in a 69B-Gal4/UAS-*FzN* wing and is most often seen in lateral anterior and posterior regions. This results in loss of margin bristles (arrow). (C,D) Margin loss is partially or completely suppressed by the co-expression of UAS-*DFz2* under 69B-Gal4 control. Conversely, the ectopic bristle phenotype of UAS-*DFz2* is suppressed by the co-expression of UAS-*DFz2N* or UAS-*FzN*, although some ectopic bristles (red arrowheads) are occasionally seen close to the margin of UAS-*FzN* wings. (E,F) Margin loss is not suppressed by the co-expression of UAS-*Fz* under 69B-Gal4 control. (G) Margin loss in a 69B-Gal4/UAS-*DFz2N* wing is modestly enhanced when dosage of *wg* is reduced by heterozygosity for the loss-of-function wg^{CX4} allele. (H) Margin loss in a 69B-Gal4/UAS-*FzN* wing is completely suppressed when dosage of *wg* is reduced by heterozygosity for the loss-of-function wg^{CX4} allele. (I,J) Bristle margin formation resulting from co-expression of UAS-*Dsh* under 69B-Gal4 control. Although UAS-*Dsh* itself severely disturbs wing patterning, margin bristles are seen along the entire margin. Ectopic bristles are observed in the wing interior (red arrows).

blocks D/V compartmentalization whereas later loss of Wg activity prevents bristle induction (Williams et al., 1994; Couso et al., 1995). If disruption of Wg signaling by the truncated proteins is the cause of the patterning defects observed in the wing, then overexpression of the full-length proteins should rescue the phenotype by titrating Wg into functional complexes. Expression of UAS-*DFz2* by 69B-Gal4 strongly suppressed DFz2N- and FzN-dependent wing margin loss (Fig. 3C,D). We also observed that DFz2N and FzN suppressed ectopic bristle induction by DFz2. These results suggest that the truncated proteins and full-length DFz2 exert opposing effects on wing outgrowth and margin bristle induction. In contrast, UAS-*Fz* had no effect on the DFz2N and FzN wing phenotypes (Fig. 3E,F).

To test whether FzN and DFz2N were interfering with Wg signaling, we reduced the *wg* gene dosage twofold and analyzed wings from flies overexpressing FzN and DFz2N. 69B-Gal4/UAS-*DFz2N* flies heterozygous for a null *wg* allele exhibited a wing phenotype that was slightly enhanced (Fig. 3G). Surprisingly, heterozygosity for *wg* suppressed the wing phenotype of 69B-Gal4/UAS-*FzN* flies (Fig. 3H). The suppression was observed with several different loss-of-function *wg* alleles (data not shown). This result argues that FzN requires Wg to inhibit wing formation, and yet it has been established that Wg is absolutely required for wing formation. The simplest interpretation is that FzN, like many dominant-negative receptors, may only inhibit endogenous signaling when it is bound to a ligand, in this case Wg. Reducing the concentration of Wg by halving gene dosage might then disable FzN if significantly less of it is in the ligand-bound state, consequently relieving its inhibitory effect on wing formation. Conversely, DFz2N may be less sensitive to Wg concentration if its affinity for Wg is much higher, possibly comparable to the endogenous Wg receptor. Halving the dosage of Wg might then equally disable both DFz2N and the endogenous Wg receptor, and the partial relief from DFz2N inhibition could be offset by a weaker interaction between Wg and its receptor. Alternatively, Wg may have a differential effect on stability of the FzN and DFz2N proteins that would account for this difference in dosage response.

If DFz2N and FzN are directly interfering with Wg signal transduction, then altering the strength of the pathway should modify the ability of DFz2N and FzN to inhibit margin bristle formation. For example, if DFz2N and FzN block Wg signaling to Dsh, then Dsh hyperactivation should suppress deletion of wing margin bristles by DFz2N and FzN. Overexpression of Dsh results in the generation of numerous ectopic bristles in the wing interior and antagonizes the loss of bristles in a *wg* mutant wing (Axelrod et al., 1996; R.W.C., unpublished data). We overexpressed Dsh and either DFz2N or FzN together in the wing using 69B-Gal4 and observed a robust bristle response along the wing margin and interior (Fig. 3I,J). This suggests that overactive Dsh can suppress bristle deletion by DFz2N and FzN. In contrast, if DFz2N and FzN are interfering with Wg signal transduction, then decreasing signal strength should enhance the ability of the truncated proteins to inhibit margin formation. The 69B-Gal4/UAS-*FzN* wing margin phenotype is enhanced when the flies are heterozygous for loss-of-function mutations in *dsh* and *arm*, another component of Wg signal transduction (Fig. 4A-C). Conversely, wing margin loss by 69B-Gal4/UAS-*FzN* is completely suppressed when flies are heterozygous for *zw3* alleles (Fig. 4D), consistent with the antagonistic role that ZW3 plays in Wg signal transduction. In summary, these genetic interactions are consistent with a model in which DFz2N and FzN block Wg signaling.

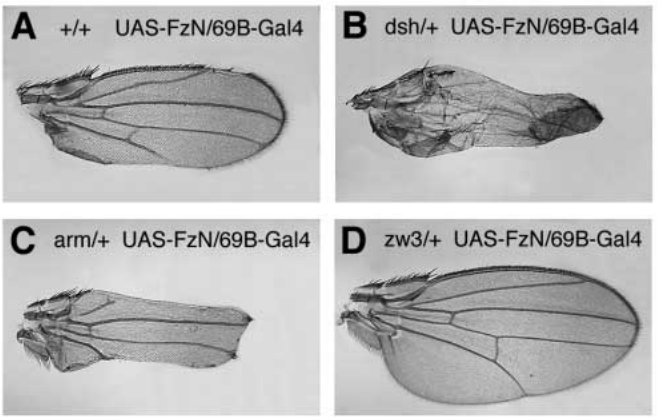

Fig. 4. FzN acts in the Wg pathway. All wings shown are from flies carrying 69B-Gal4 and UAS-*FzN*. The genetic backgrounds are: (A) wild type; (B) dsh^{v26}/+; (C) arm^{xk22}/+; (D) $zw3^{m11}$/+. Note that wing formation is severely inhibited in 69B-Gal4/UAS-*FzN* flies carrying one copy of a *dsh* or *arm* loss-of-function allele (B,C). Wing formation is rescued by the presence of one copy of a *zw3* loss-of-function allele (D).

Wg is synthesized in the ventral compartment during early wing development, where it functions to specify the wing primordium and to activate transcription of the *vestigial* (*vg*) gene in D/V boundary cells through a boundary-specific enhancer (Williams et al., 1994; Couso et al., 1995). We examined the level of *vg* enhancer activity using a *lacZ* reporter transgene containing the *vg* boundary-specific enhancer element (Williams et al., 1994). In 69B-Gal4/UAS-*FzN* wing discs, the level of β-galactosidase is reduced along the D-V boundary, suggesting that FzN interferes with *vg* enhancer activity (Fig. 5A,B). Establishment of the D/V boundary precedes a later pattern of Wg expression which is within a stripe of cells at the D/V boundary (Diaz-Benjumea and Cohen, 1995). The Apterous (Ap) transcription factor is required to establish this later pattern of Wg expression (Cohen et al., 1992; Kim et al., 1995). We examined the expression of Ap and Wg in late third-instar wing discs of FzN larvae using *ap-lacZ* and *wg-lacZ* reporter genes. The expression patterns of *lacZ* in wild type and 69B-Gal4/UAS-*FzN* are indistinguishable, suggesting that FzN blocks wing margin development downstream of Ap and Wg (Fig. 5C,D; data not shown).

In response to Wg signaling from the D/V boundary, proneural genes such as *achaete* (*ac*) and *neuralized* (*neu*) are expressed in cells adjacent to the D/V boundary. Proneural gene activation is essential for development of sensory bristles along the wing margin (Phillips and Whittle, 1993). Expression of *ac* and *neu* are abolished in cells on both sides of the D/V boundary in 69B-Gal4/UAS-*FzN* wing discs (Fig. 5E-H). To relate the domain of FzN expression more precisely to its inhibitory activity, a Dpp-Gal4 driver line was used to specifically express FzN along the anterior/posterior (A/P) midline of the wing disc (Fig. 6A). Expression of endogenous

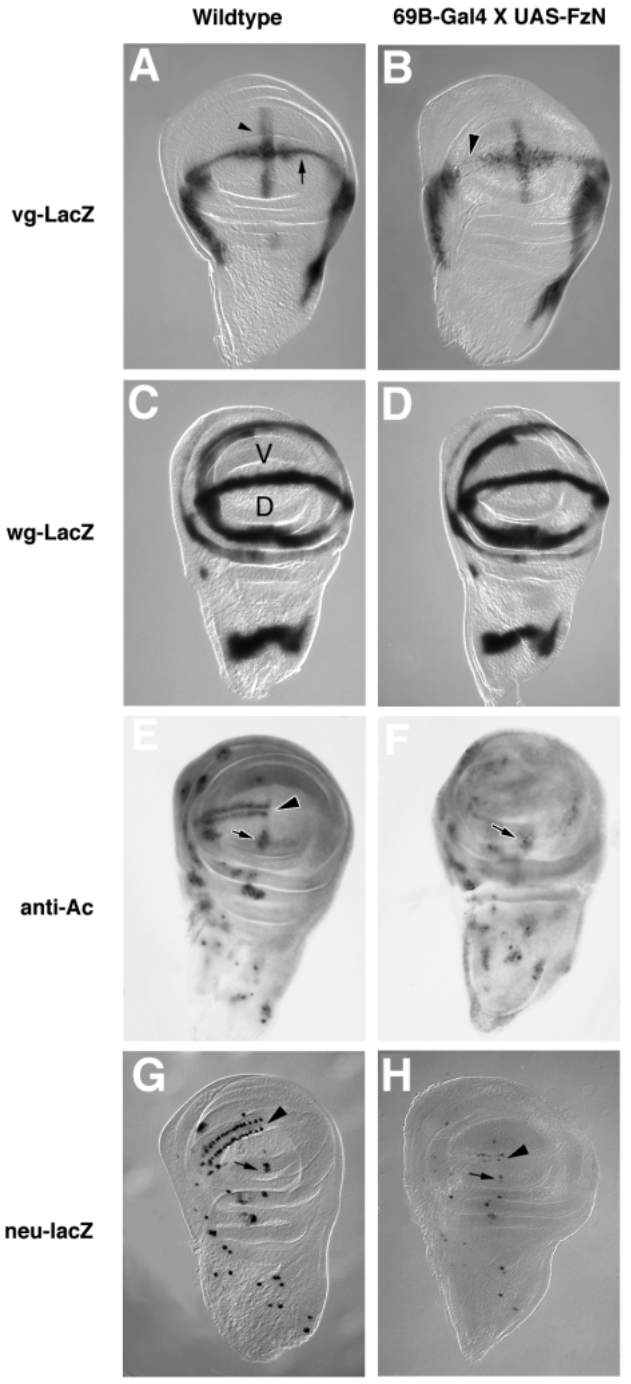

Fig. 5. FzN inhibits the activation of Wg downstream genes. The expression of *wg* and its target genes is compared between wild-type (A,C,E,G) and 69B-Gal4/UAS-*FzN* animals (B,D,F,H). (A) X-gal staining of a third instar wild-type wing disc with *lacZ* expression directed by a *vg* boundary-specific enhancer element. The enhancer activity is detected along the D-V boundary (arrow) as well as the A-P boundary (arrowhead). (B) *vg-lacZ* expression is lost (arrowhead) along the D-V boundary in several places in the 69B-Gal4/UAS-*FzN* wing disc. (C) Wild-type *wg* expression pattern as indicated by a *wg-lacZ* reporter gene expression. The *wg* expression straddles the D-V boundary and wing pouch boundary. (D) The expression of *wg-lacZ* in 69B-Gal4/UAS-*FzN* wing disc is indistinguishable from that of the wild type. (E) Ac protein is present in the wild-type wing disc as two parallel rows (arrowhead) along the anterior D-V boundary. (F) Ac protein is not detected along the D-V boundary in a 69B-Gal4/UAS-*FzN* disc. Ac expression in vein sensilla clusters (arrow) persists in the disc, albeit at a lower level. (G) *neu-lacZ* expression marks the neuronal clusters along the anterior D-V boundary (arrowhead) and other sensory neuron precursors (arrow) of a wild-type wing disc. (H) *neu-lacZ* expression is reduced in a 69B-Gal4/UAS-*FzN* wing disc.

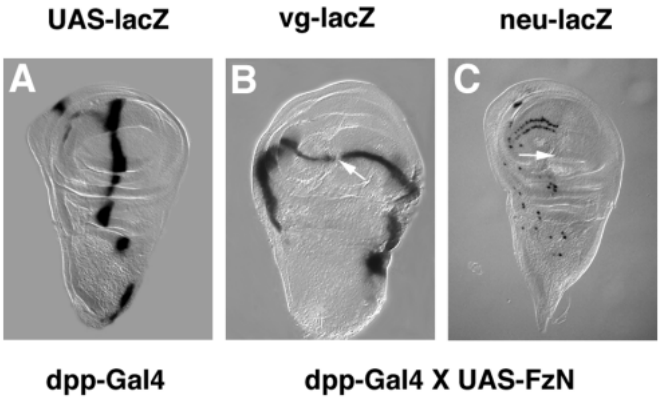

Fig. 6. The inhibitory effect of FzN co-localizes with its expression. (A) Dpp-Gal4 is expressed along the A-P compartment boundary as revealed by X-gal detection of a UAS-*lacZ* reporter gene. Expression of UAS-*FzN* using the Dpp-Gal4 driver causes specific inhibition of *vg-lacZ* (B) and *neu-lacZ* (C) expression along the A-P boundary only. In B and C, compare expression of the reporter genes in the regions indicated with arrows to the expression in comparable regions of wild-type discs (Fig. 5A,G)

A/P boundary genes such as *dpp* and *engrailed* (*en*) were not affected (data not shown). Activity of the *vg* boundary enhancer and expression of *neu* were specifically blocked along the A/P midline (Fig. 6B,C), indicating that FzN inhibitory activity correlates with its expression domain.

Wg signaling is also known to function in the embryonic ectoderm to direct cell fates, promoting differentiation of cells that secrete smooth cuticle rather than small hairs called denticles. Embryos with reduced Wg activity fail to produce smooth cuticle and instead have a continuous lawn of denticles (Bejsovec and Martinez-Arias, 1991). We tested whether FzN blocks Wg signaling in embryos by expressing UAS-*FzN* with Patched-Gal4 and 69B-Gal4 drivers. In these embryos, fusion of denticle belts was observed between some segments indicating a decrease in the amount of smooth cuticle (Fig. 7B,D). Sometimes a mirror image duplication of an entire denticle belt was observed (Fig. 7C). The phenotypes are similar to those produced by weak loss-of-function *wg* alleles. Together with the wing margin phenotypes, this result suggests that removal of the intracellular sequences of Fz or DFz2 interferes with Wg activity in various tissues.

DISCUSSION

DFz2 acts in the Wg pathway in vivo

The view that members of the Frizzled family of proteins are central in transmembrane signaling by Wnts in part stems from

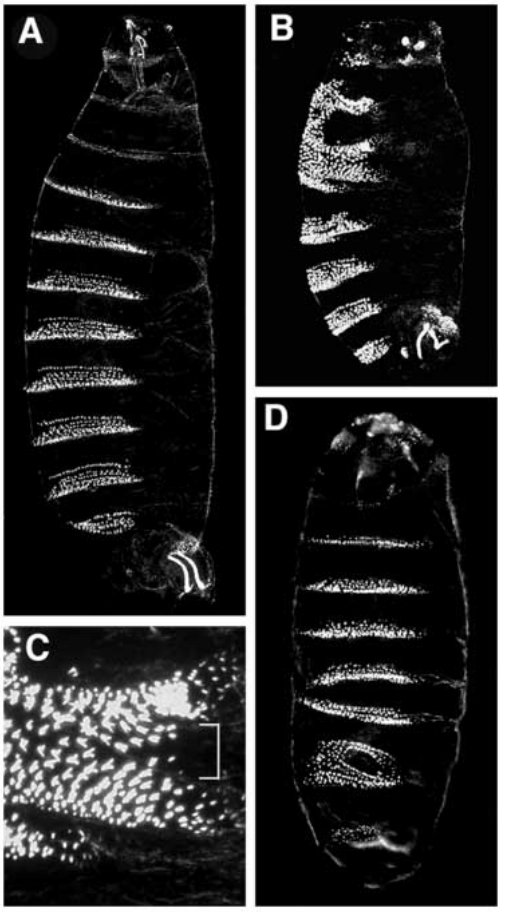

Fig. 7. FzN affects embryonic segmental polarity. (A) Cuticle of an unhatched wild-type embryo (*Patched-Gal4/+*). Note that denticle bands alternate with naked cuticle within each segment. (B,C) Cuticle of an unhatched Patched-Gal4/UAS-*FzN* embryo. Naked cuticle is deleted in some segments and is replaced by denticle belts. There are also defects in the head and tail. Note that the posterior segmental region (indicated by bracket in C) is covered by denticles with reversed polarity, often pointing toward the midline. (D) Cuticle of an unhatched 69B-Gal4/UAS-*FzN* embryo.

the binding activities of these proteins. Wg proteins bind to *Drosophila* or human tissue culture cells expressing either Fz or DFz2 (Bhanot et al., 1996). Moreover, DFz2 expressed in tissue culture cells promotes stabilization of Arm in response to Wg. Here, we extend this experimental evidence and find that DFz2 has the expected properties of acting in the Wg pathway to induce wing margin bristles. First, we find that overexpression of DFz2 produces ectopic bristles at a greater distance from Wg-secreting cells along the wing margin than normal, and this occurs in a Wg-dependent manner. Formation of ectopic bristles is suppressed by halving the dosage of Wg. Second, we find that a dominant-negative form of DFz2 blocks Wg signaling, and this effect is specifically suppressed by overexpressing full-length DFz2. Third, we find that the dominant-negative form of DFz2 suppresses ectopic bristle formation by DFz2, consistent with the hypothesis that DFz2 acts in the Wg pathway.

How does the overexpression of DFz2 lead to ectopic bristle induction? One possibility is that cells at some distance from the narrow stripe of Wg-secreting margin cells are normally exposed to a low level of Wg, insufficient to induce bristle determination. Overexpression of DFz2, combined with endogenous receptors, may increase Wg signal transduction in some of these cells to a level comparable to those nearer the wing margin, thus inducing ectopic margin bristles. It is worth noting that at the anterior margin, most ectopic bristles induced close to the margin are of the stout mechanosensory type which are normally present in a row of cells closest to the stripe of Wg secretion. The ectopic bristles further away from the wing margin are usually of the slender or chemosensory types which are normally present in two rows of cells more distant from the Wg stripe than the stout row. This suggests that Wg is normally present in the wing as a concentration gradient surrounding the wing margin. Overexpression of DFz2 increases the level of Wg signal transduction in cells that are exposed to a particular concentration of Wg. Consequently, cells adopt fates which are normally induced only at a higher concentration of Wg. Cells that would have normally adopted slender or chemosensory bristle fates, instead become stout bristles, and cells that would have normally failed to adopt any bristle fate, instead become slender or chemosensory bristles. This interpretation is consistent with observations that Wg acts as a gradient morphogen to induce expression of different target genes near the wing margin (Zecca et al., 1996).

Evidence from us and others indicate that Fz does not likely function in Wg signaling in wing margin development. Comparison of loss-of-function *wg* and *fz* wing phenotypes indicate that the two genes have non-overlapping activities (Gubb and Garcia-Bellido, 1982; Struhl and Basler, 1993). From our experiments, we found that overexpression of Fz does not perturb Wg signaling nor does a reduction in Wg dosage modify the polarity phenotypes generated by overexpressed Fz. Moreover, the dominant-negative block of Wg signaling by DFz2N is not rescued by overexpressing Fz. However, previous observations in other tissues suggest that Fz and Wg may be functionally linked. First, overexpression of Fz phenocopies overexpression of Wg in embryos (Tomlinson et al., 1997). Second, clones of retinal cells that constitutively express Wg induce polarity inversions in nearby ommatidia of the eye (Treisman and Rubin, 1995; Tomlinson et al., 1997). However, other experiments on retinal polarity have suggested that Wg indirectly defines polarity by specifying the eye's D/V midline (Reifegerste et al., 1997; Heberlein et al., 1998). The midline consequently generates a Fz-dependent signal to differentiating photoreceptor cells that direct ommatidial polarity (Zheng et al., 1995). Wg does not appear to be this later signal since late removal of Wg activity does not affect polarity (Treisman and Rubin, 1995; Ma and Moses, 1995). Moreover, misexpressing Wg in the *sevenless* expression pattern of differentiating retinal cells has no effect on polarity (Cadigan and Nusse, 1996). In this regard, it is not surprising that our misexpression of DFz2 in the *sevenless* expression pattern does not affect ommatidial polarity if Wg does not normally signal to differentiating retinal cells. It is unclear which, if any, Wnt interacts with Fz. Misexpression of the other identified *Drosophila* Wnts (Wnt-2,3,4) in the imaginal discs has no effect on polarity (data not shown).

DFz2N and FzN are dominant-negative receptors for Wg

It appears that truncated DFz2 and Fz proteins have dominant-negative activities affecting Wg signal transduction. Their overexpression produces phenotypes in diverse tissues that closely resemble loss-of-function *wg* mutants. The expression of Wg and genes acting upstream of Wg are not affected but expression of target genes downstream of Wg are inhibited. Finally, the phenotypes produced are sensitive to the dosage of genes acting in the Wg signal transduction pathway.

There are two potential caveats to our interpretation that truncated Fz and DFz2 act as a dominant-negative receptors for Wg. First, it is possible that they block the activity of a pathway other than that of Wg, such as the Notch pathway which also functions in formation of the D/V wing boundary and margin structures (Diaz-Benjumea and Cohen, 1995; Neumann and Cohen, 1996). However, Wg expression at the D/V wing boundary requires Notch activity and yet Wg expression is not altered in FzN flies, indicating that FzN does not inhibit Notch activity. Moreover, FzN does not block lateral inhibition of bristle development, which requires Notch but occurs independently of Wg (Cabrera, 1990). Altogether, it is unlikely that the Notch pathway is significantly affected by truncated DFz2 or Fz.

A second caveat is that the inhibition of *vg* expression by FzN argues against a recently proposed model for *vg* regulation (Neumann and Cohen, 1996). According to this model, *vg* expression is initially activated in cells at the D/V boundary by a Notch-dependent signal which acts through the boundary-specific enhancer. Wg does not appear to directly regulate this phase of *vg* expression. However, we observe that *vg* boundary-specific expression is inhibited by the constitutive presence of FzN, in direct conflict with this model. The model also conflicts with evidence in which boundary-specific *vg* expression was lost when Wg was absent in wing discs during second instar (Williams et al., 1994). Normally *vg* expression is localized to the wing pouch and not to regions of the wing disc fated to become body wall. Since early loss of Wg signaling causes cells in the wing pouch to adopt a body wall fate rather than wing identity, such transformed cells may be unable to express *vg*. Possibly FzN interferes with this early phase of Wg signaling and causes a partial fate transformation. Consistent with this interpretation, the *dpp* and *en* genes are expressed in both wing pouch and body wall cells, and expression of neither gene is affected by FzN.

The mechanism by which truncated DFz2 and Fz inhibit Wg signaling is unclear. They might sequester the Wg ligand from binding to its endogenous receptor. Since similar truncated versions have been demonstrated to bind Wg in vitro (Bhanot et al., 1996), it is likely that this occurs in vivo as well. It is also consistent with the finding that reduced *wg* gene dosage potentiates the response to DFz2N. Moreover, secreted Frzb proteins in vertebrates contain a domain similar to the Frizzled-family extracellular domain, and Frzb proteins antagonize Wnt signaling by binding and inactivating Wnts (Leyns et al., 1997; Wang et al., 1997a). However, our observation that reducing *wg* gene dosage suppresses the FzN phenotype suggests that Wg is not being titrated in these animals to a level that is severely limiting for its activity. Instead, this observation suggests that FzN must interact with Wg in order to exert its dominant-negative effect, which may be to sequester another component in the pathway. Indeed, dominant-negative forms of growth factor receptors with a similar architecture to FzN behave in this manner (Kashles et al., 1991; Ueno et al., 1992). For example, a mutant EGF receptor lacking a cytoplasmic domain binds to EGF and subsequently forms nonproductive heterodimers with wild-type receptors (Kashles et al., 1991). Similarly, ligand-bound truncated Fz or DFz2 might form nonfunctional heterodimers with endogenous Wg receptors or other receptor complex components and inhibit their transducing activities. It is unlikely that a cytoplasmic component of the signal transduction pathway is titrated since the small cytoplasmic domains of DFz2N and FzN are structurally unrelated to any Fz protein.

Dominant-negative DFz2 and Fz proteins have relaxed specificity

We have found that full-length DFz2 and not Fz possesses the expected properties of the Wg receptor in wing margin

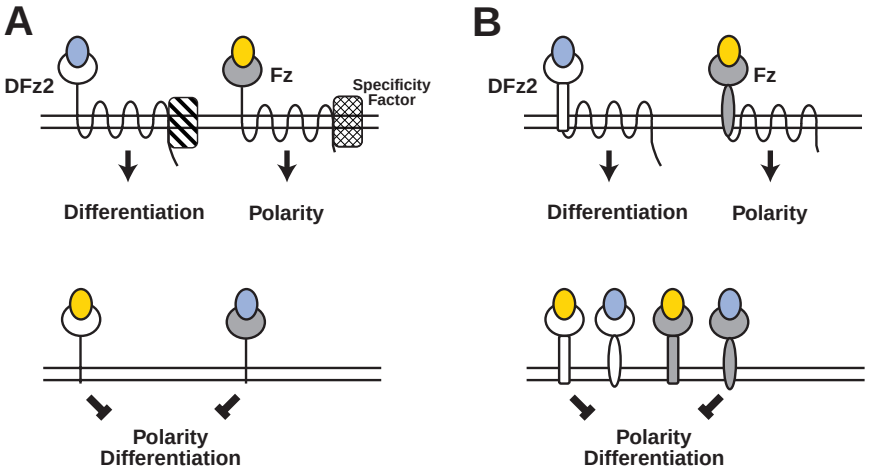

Fig. 8. Possible models for DFz2 and Fz signal transduction. (A) Specificity factors associate with either DFz2 or Fz (shown at the top) and regulate their ability to transduce a signal. One factor would restrict DFz2 to transducing a differentiation signal in response to Wg, and another factor would restrict Fz to transducing a polarity signal in response to a different ligand. If these factors associate with a region distal to the first transmembrane domain, then they would be unable to associate with either DFz2N or FzN (shown at the bottom). Consequently, DFz2N and FzN would be unrestricted in their abilities to suppress both differentiation and polarity. (B) A specific conformation of DFz2 and Fz (shown at the top) restricts each protein to transduce a specific signal. The DFz2 conformation would restrict it to transducing a differentiation signal in response to Wg, and the Fz conformation would restrict it to transducing a polarity signal. Truncation of these proteins to produce DFz2N and FzN would relax their conformational specificity such that either truncated protein could adopt either conformation. Consequently, DFz2N and FzN would be unrestricted in their abilities to suppress both differentiation and polarity.

development. However, truncated forms of both DFz2 and Fz disrupt Wg signaling. This difference in specificity between full-length and truncated Fz proteins is exemplified by the inability of full-length Fz to rescue the dominant-negative patterning phenotype of truncated Fz. Moreover, truncated forms of Fz and DFz2 disrupt polarity signaling in the eye, but only full-length Fz and not DFz2 appears to affect polarity signaling. What might account for these differences? One possibility is that a specificity factor associates with each full-length protein and restricts signal transduction to a unique Wnt pathway (Fig. 8A). If the specificity factor interacts with a region distal to the first transmembrane domain, then it would be unable to associate with the truncated proteins and restrict their activities to a single pathway. Another possibility is that the conformation of each full-length protein restricts signal transduction to a unique Wnt pathway (Fig. 8B). This conformational specificity would be relaxed in proteins truncated at the first transmembrane domain, permitting their promiscuous interaction with components of multiple Wnt pathways. For both models, the full-length proteins could either be restricted in their association with an effector or in their Wnt-binding specificity. We favor the former possibility since full-length Fz has been demonstrated to interact with Wg in S2 cells (Bhanot et al., 1996). Moreover, the secreted Frzb-1 protein specifically inhibits activity of a subset of Wnts during *Xenopus* embryogenesis (Wang et al., 1997b).

Use of dominant-negative receptors has been a powerful genetic approach to study cytokine signaling in a variety of organisms, including ones not amenable to traditional genetics (Kashles et al., 1991; Rebay et al., 1993; Cornell and Kimelman, 1994; Cascino et al., 1996; Massague, 1996). A basic precept of these approaches has been that the dominant-negative receptors retain their native specificity for ligand or effector. Consequently, they can functionally ablate specific signaling pathways while not affecting other related pathways. We have discovered that a truncated version of the Fz family of receptors does not possess this degree of specificity and blocks more than one signaling pathway. It is unclear whether loss of specificity is particular to the location of the deletion endpoint in the truncated protein or is a general feature of this family of proteins. Nevertheless, it points out the possibility that using a dominant-negative Fz receptor could block more developmental processes than those that are normally associated with the full-length receptor.

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