Wingless transduction by the Frizzled and Frizzled2 proteins of Drosophila

Chiann-mun Chen and Gary Struhl*

Howard Hughes Medical Institute, Department of Genetics and Development, Columbia University College of Physicians and Surgeons, New York, NY 10032, USA
*Author for correspondence (e-mail: struhl@cuccfa.ccc.columbia.edu)

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SUMMARY

Wingless (Wg) protein is a founding member of the Wnt family of secreted proteins which have profound organizing roles in animal development. Two members of the Frizzled (Fz) family of seven-pass transmembrane proteins, Drosophila Fz and Fz2, can bind Wg and are candidate Wg receptors. However, null mutations of the fz gene have little effect on Wg signal transduction and the lack of mutations in the fz2 gene has thus far prevented a rigorous examination of its role in vivo. Here we describe the isolation of an amber mutation of fz2 which truncates the coding sequence just after the amino-terminal extracellular domain and behaves genetically as a loss-of-function allele. Using this mutation, we show that Wg signal transduction is abolished in virtually all cells lacking both Fz and Fz2 activity in embryos as well as in the wing imaginal disc. We also show that Fz and Fz2 are functionally redundant: the presence of either protein is sufficient to confer Wg transducing activity on most or all cells throughout development. These results extend prior evidence of a ligand-receptor relationship between Wnt and Frizzled proteins and suggest that Fz and Fz2 are the primary receptors for Wg in Drosophila.

Key words: Frizzled, Wingless, Signal transduction, Drosophila melanogaster

INTRODUCTION

Wingless/int1 (Wnt) proteins are secreted glycoproteins that have diverse and profound roles in animal development (reviewed by Wodarz and Nusse, 1998; Dierick and Bejsovec, 1999). Wnt proteins act both as short range inducers and long range morphogens. For example, during Drosophila embryogenesis, Wingless (Wg) secreted by cells located just anterior to the parasegment boundary within each segment directs adjacent cells just posterior to the boundary to maintain expression of the selector gene engrailed (en) (DiNardo et al., 1988; Martinez Arias et al., 1988; Bejsovec and Martinez Arias, 1991; Heemskerk et al., 1991; Vincent and Lawrence, 1994). Later in development, Wg is secreted by defined sub-populations of cells within the primordia forming the adult appendages and acts directly on cells over a range of 20-30 cell diameters to organize their patterns of gene expression and cuticular differentiation (Struhl and Basler, 1993; Zecca et al., 1996; Neumann and Cohen, 1997; Cadigan et al., 1998).

Many conserved components of the cellular machinery responsible for transducing Wnt proteins have been identified, including the cytosolic proteins Dishevelled (Dsh), Glycogen Synthase Kinase 3 (GSK-3/Zeste-white3/Shaggy), Axin, APC, Slimb and Armadillo (Arm/β-catenin), as well as a transcription factor LEF (LEF/TCF/Pangolin) (reviewed by Wodarz and Nusse, 1998; Dierick and Bejsovec, 1999). These proteins appear to define an intracellular signal transduction pathway leading from reception of Wg at the cell surface to the activation of target genes in the nucleus.

The identity of the cell surface receptor that links Wg to this intracellular signal transduction pathway has been less certain. In the past three years, proteins of the Frizzled (Fz) family have become the leading candidates for the Wnt receptors. Fz proteins contain a large extracellular N terminus containing a conserved cysteine-rich domain (CRD) followed by seven transmembrane domains and a small cytosolic C terminus (Wodarz and Nusse, 1998). In Drosophila, attention has been focused principally on two Fz proteins, Fz and Fz2.

The first indication that Fz proteins might serve as Wnt receptors came from the discovery that Drosophila Fz2 can confer Wg binding activity in tissue culture cells as well as ectodermal cells in vivo (Bhanot et al., 1996; Nusse et al., 1997; Cadigan et al., 1998). Fz itself was also shown to confer Wg binding activity in tissue culture (Bhanot et al., 1996; Nusse et al., 1997; Cadigan et al., 1998). These binding studies are consistent with a function for Fz proteins as Wnt receptors, but do not in themselves establish a ligand-receptor relationship.

One genetic approach, overexpression of wild-type or truncated forms of Fz proteins, has provided additional support for such a relationship. Overexpression of either Fz or Fz2 in Drosophila causes phenotypes associated with ectopic Wg signaling (Cadigan and Nusse, 1997; Tomlinson et al., 1997; Zhang and Carthew, 1998), whereas overexpression of membrane tethered forms of the extracellular Fz2 CRD (the putative Wg-binding domain) causes phenotypes associated with attenuated Wg signaling (Bhat, 1998; Cadigan et al., 1998; Zhang and Carthew, 1998). Similar results have been obtained in vertebrates; vertebrates also express a class of Fz-
related proteins composed of only the extracellular domain that appear to function as endogenous antagonists of Wnt signaling (reviewed by Wodarz and Nusse, 1998; Dierick and Bejsovec, 1999). However, these results do not show that Fz proteins are normally required for transducing Wnt signals.

If Fz proteins function as Wnt receptors, then their activities should be essential for Wnt signal transduction. However, to date, loss-of-function phenotypes have not established that this is so. Mutations that abolish activity of Drosophila Fz, the founding member of the Fz family, have revealed that Fz is required for the normal planar polarity of epithelial cells (Gubb and Garcia-Bellido, 1982; Vinson and Adler, 1987; Zheng et al., 1995). However, the nature of the polarizing signal is not known, and fz mutant animals appear to transduce Wg normally. Concomitant reduction of Fz and Fz2 activity by RNA-mediated interference, or by the use of large genomic deletions which eliminate zygotic, but not maternal, gene activity during embryogenesis can cause phenotypes resembling those associated with reduced Wg signalling (Bhat, 1998; Kennerdell and Carthew, 1998; Muller et al., 1999). However, the reported phenotypes are less severe than those caused by the absence of Wg itself, leaving open the question of whether Fz proteins are essential for Wg signal transduction.

A major impediment in resolving whether Drosophila Fz proteins function as Wnt receptors has been the lack of a loss-of-function mutation in fz2, which has been proposed to encode the primary Wg receptor (Bhanot et al., 1996; Cadigan et al., 1998). The absence of such a mutation makes it difficult to assess whether either Fz or Fz2 can transduce Wg, and if so, whether they are functionally redundant or play distinct roles, e.g., in transducing Wg in different contexts, or in transducing additional Wnts. It also hinders the assessment of other hypotheses about Wg signal transduction, such as the suggestion that Wg signaling might involve other receptors or coreceptors, e.g., Notch (Couso and Martinez Arias, 1994) or proteoglycans (Reichschman et al., 1996; Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997; Lin and Perrimon, 1999; Tsuda et al., 1999), or that the movement or stability of secreted Wg may depend on the distribution of its receptors (Cadigan et al., 1998).

Here, we describe the isolation of a likely null allele of the Drosophila fz2 gene and examine the consequences of removing either or both the fz and fz2 gene functions during development. Our main finding is that the elimination of both Fz and Fz2 activity causes an absolute loss of Wg transduction in virtually all of the Wg signaling processes that we have assayed, both in embryos and in the developing primordium of the adult wing. In contrast, absence of either Fz or Fz2 activity alone, has little if any detectable effect on Wg signaling in any tissue at any stage of development. We conclude that there is an absolute requirement for Fz proteins in transducing Wg in most or all developmental contexts, but that either Fz or Fz2 can carry the full burden of Wg signal transduction on its own. These results, taken together with previous findings, lead us to propose that Fz and Fz2 are the primary receptors for Wg in Drosophila.

MATERIALS AND METHODS

Mutations

The following null or amorphic alleles for fz, wg, and dsh were used: fz1 H51 (Jones et al., 1996), wg CX4 (Baker, 1987) and dsh25 (Manoukian et al., 1995). Other mutations are described in Flybase (http://flybase.bio.indiana.edu/).

Transgenes

The following Gal4 driver lines were used. vg Ox26-Gal4 (Gal4 expressed under the control of the vestigial boundary enhancer along the dorsoventral compartment boundary of the wing imaginal disc, Simmonds et al., 1995; originally developed by S. Morimura and M. Hoffman). dpp Gal4 (Morimura et al., 1996). h Gal4 (Brand and Perrimon, 1993).

The following FLP-out transgenes were generated by standard methods using previously described transgenes (Brand and Perrimon, 1993; Basler and Struhl, 1994; Nellen et al., 1996; Zecca et al., 1996) as starting materials and the fz2, fz2 C1 and GFP coding sequences (Bhanot et al., 1996; details available upon request). Tubulin1 > CD2, y > fz2. UAS>GFP, y > fz2. UAS>CD2, y > fz2 C1 (the fz2 C1 coding sequence was introduced by replacing the wild-type coding sequence from the Apal site to the termination codon with the corresponding genomic DNA of the mutant allele, from Apal to the newly induced Xbal site which introduces a termination codon). In most cases, the FLP-out cassettes in these transgenes were removed prior to the experiment.

Additional transgenes employed are as follows. FRT2A (Perrimon et al., 1996). hsp70-flp UAS>CD2, y >flu-AArm and UAS>CD2, y >flu-wg (Zecca et al., 1996). hsp70-GFP and hsp70-CD2, y > insered on 3L; G.S. and A. Adachi, unpublished materials, see also Jiang and Struhl, 1995, 1998). UAS-flop (generated by inserting the flop coding sequencing into the pUAST vector; Brand and Perrimon, 1993). The OvoD.w+ transgene, and its use in generating female germ line clones are described in Perrimon et al. (1996).

Genetic screen

Males of the genotype y; fzl H51 fzl2/fz2 TM2 were mutagenized with EMS and crossed to females of the genotype y; vg Ox26/Gal4 UAS-flp; hs CD2, y > fzl2/TM2.

F1 progeny of the genotype y; vg Ox26/Gal4 UAS-flp/+; fzl H51 fzl2/R2A hs CD2, y > fzl2/TM2 express Flp primarily in wing imaginal cells under vg Ox26/Gal4 control, especially in cells giving rise to the margin. This Flp activity mediates a high frequency of mitotic recombination (Golic, 1991), generating clones of cells homozygous for the mutated fz1 H51 fzl2 R2A chromosome arm. The wings of the resulting Fl1 flies were screened for wing notches and ecotropic margin bristles. Approximately 24,000 F1 flies were screened, leading to the identification of a single candidate fzl2 mutation, fzl2 C1 (see Results). We have subsequently observed that animals of the same genotype as the fly in which the fzl2 C1 mutation was initially identified; y; vg Ox26/Gal4 UAS-flp/+; fzl H51 fzl2 C1 fzl2/R2A hs CD2, y > fzl2/TM2, are less fit than wildtype flies, probably because of leaky expression of the UAS-flp transgene, and consequently the generation of fzl H51 fzl2 C1 clones, in other tissues. The lack of fitness associated with this genotype may account for only one fzl2 mutation being obtained.

Verification of the fzl2 C1 mutation

The fzl2 C1 mutation was mapped relative to the ri and fzl loci by standard genetic methods. The UAS>y > fzl2 and Tubulin1 > fzl2 transgenes were each introduced together with the fz1 H51 fzl2 C1 /fzl2 R2A chromosome under the conditions of the screen to assay for the rescue of the wing notching phenotype associated with the double mutant clones. Genomic DNA was obtained from fz1 H51 fzl2 C1 embryos derived from fz1 H51 fzl2 C1 germline clones, amplified by PCR, subcloned and sequenced. The fzl2 C1 allele is associated with a TGG to TAG mutation in codon Trp 320 which introduces an Xbal site. This restriction site polymorphism was used subsequently to confirm the genotype of fzl2 C1/fzl2 C1 flies.

As noted in the Results, fzl2 C1/fzl2 C1 flies derived from heterozygous parents differ from their heterozygous siblings in several respects. First, they are usually developmentally delayed, typically eclosing as adults 1-2 days later, and show variable survival to the adult, depending on genetic background. Second, they tend to be smaller in size, although...
normally proportioned and patterned. Finally, they are sterile both as males and females. None of these phenotypes resemble phenotypes known to be associated with reductions in Wg signaling and it is possible that they are not due to the mutation itself, but rather to second site mutations on the same chromosome that are unrelated to the lesion in the \( f_{z2} \) gene. Consistent with this possibility, these phenotypes do not appear to be fully rescued by the presence of a Tubulin\(\alpha\)-\(f_{z2}\) transgene. We also note that \( f_{z2}^{C1} \) homozygotes derived from mutant \( f_{z2}^{C1} \) eggs hatch as morphologically normal first instar larvae (Fig. 6E), but die at varying times during larval development.

**Generation of clones for phenotypic analysis**

Eggs from \( f_{z1}^{H51} f_{z2}^{C1} \) or \( f_{z2}^{C1} \) germ-line cells were obtained using the FLP/FRT/ovoD method (Chou et al., 1993). Females carrying these clones were fertilized by \( f_{z1}^{H51} f_{z2}^{C1}/TM3, fz-lacZ, f_{z2}^{H51}/TM3, fz-lacZ, \) or \( f_{z2}^{C1}/TM3, fz-lacZ \) males, depending on the experiment, allowing the various mutant and wild-type progeny to be distinguished by staining for Fz-\(\beta\)-gal expression. Individual clones of \( f_{z2}^{C1} \) mutant cells marked by the absence of the hsp70-GFP marker gene were generated using the FLP/FRT method (Golic, 1991; Chou et al., 1993; Xu and Rubin, 1993) as follows. Larvae of the genotype \( y^{hs70-flp; f_{z2}^{H51} f_{z2}^{C1}} \) or \( f_{z2}^{C1} \), \( r^{fRT2A/hsp70-C2D, y^{hs70}} \), hsp70-GFP FRT2A were heat shocked for 1 hour at 37°C during the first instar, or at 35°C during the early- to mid- third instar to induce the mutant clones. They were subsequently subjected to a second 37°C heat shock for 60 minutes and allowed to recover for 1 hour at 25°C before fixation and immunostaining to induce expression of the hsp70-GFP marker gene. For the experiment involving the Minute technique, the \( M(3)i^{ts} \) mutation was introduced on the hsp70-C2D, y* hsp70-GFP FRT2A chromosome.

**Overexpression and epistasis experiments**

For the overexpression experiment, imaginal wing discs derived from larvae of the genotype \( UAS>f_{z2}^{H51} dpp-G4 \) were stained for Wg and Fz2 expression, and the resulting adults assayed for alterations in normal patterning. For the epistasis experiment, females carrying \( h-Gal4 f_{z2}^{H51} \) \( f_{z2}^{C1} \) germline clones were crossed to males of the genotype \( UAS📅f_{z2}^{H51} \) \( f_{z2}^{C1} Arm \) (or \( UAS一万u-wg\)) \( f_{z1}^{H51} f_{z2}^{C1}/TM3, fz-lacZ \) and the resulting first instar larvae analyzed for defects in cuticular pattern. Under these conditions, approximately half of the embryos inherit the \( TM3, fz-lacZ \) chromosome and develop into phenotypically wild-type larvae, whereas the remaining half inherit the \( UAS📅f_{z2}^{C1} \) (or \( UAS一万u-wg\)) \( f_{z1}^{H51} f_{z2}^{C1} \) chromosome and show the phenotypes illustrated in Fig. 2D,E.

**Antibody staining**

Standard protocols for immunohistochemistry and immunofluorescence were followed for both embryos and imaginal discs (Struhl and Basler, 1993; Zecca et al., 1996) using mouse anti-Wg, mouse anti-\(\text{En}\), mouse anti-Arm, rabbit anti-Vg, rabbit anti-Fz2, mouse anti-Dll, rabbit anti-Eve, rabbit anti-GFP (Clontech), and rabbit anti-\(\beta\)-gal (Cappel) antisera (Diederich et al., 1989; Wu et al., 1995; Bhanot et al., 1996; Kim et al., 1996; Zecca et al., 1996; Neumann and Cohen, 1997; and references therein). The Armadillo mAb N27A1, En mAb 4D9 and Wg mAb 4D4 antisera developed by E. Wieschaus, N. Patel and S. Cohen were obtained from the Developmental Studies Hybridoma bank, Department of Biological Sciences, Iowa City, IA.

**RESULTS**

**Isolation of a loss-of-function mutation in the Drosophila \( f_{z2} \) gene**

Previous saturation genetic screens for both maternal and zygotic factors required for normal embryonic patterning have failed to identify any mutations in the \( f_{z2} \) gene, despite yielding multiple mutant alleles of several other genes involved in Wg signaling, including Wg itself (Nüsslein-Volhard and Wieschaus, 1980; Perrimon et al., 1989, 1996). The failure to identify \( f_{z2} \) mutations in these screens might indicate that Fz2 is not involved in Wg signal transduction. However, it is also possible that a requirement for Fz2 activity would only be apparent when both maternal and zygotic contributions are eliminated, or in the absence of other potentially redundant activities, particularly Fz.

Recessive, loss-of-function mutations in \( f_{z2} \) cause defects in planar polarity of epithelial cells: mutant animals are viable and generally display a normal morphology except that the bristles and hairs secreted by epidermal cells point in abnormal directions. However, they do not appear to compromise Wg signal transduction. To assess the possibility that Fz and Fz2 have redundant roles in Wg signal transduction, we conducted a screen for new, recessive mutations that block Wg signal transduction in \( f_{z2} \) mutant cells. Conveniently, both the \( f_{z2} \) and \( f_{z2} \) genes are located on the left arm of the third chromosome. This allowed us to conduct an “\( F_{1} \)” genetic screen in which flies carrying clones of wing cells homozygous for a \( f_{z2} \) mutation and newly induced, second site mutations on this chromosome arm were screened for phenotypes reflecting a loss in Wg signal transduction (Materials and Methods).

Wg is normally expressed in a thin stripe of cells straddling the dorsoventral compartment boundary of the mature wing imaginal disc, under the control of the extracellular signals Delta and Serrate (reviewed by Irvine and Vogt, 1997). Wg emanating from these cells directs the formation of wing margin bristles and organizes gene expression, growth and patterning in surrounding cells of the presumptive wing blade (Zecca et al., 1996; Neumann and Cohen, 1997). Hence, mutations that block Wg signal transduction cause a loss of wing margin bristles as well as deletions of nearby portions of the wing. Wg also plays a role in restricting its own expression to cells immediately adjacent to the dorsoventral compartment boundary, down-regulating the transcription of wgl itself in neighboring cells that are close, but not next, to the D/V boundary (Rulifson et al., 1996). When Wg signal transduction is blocked in these cells, they ectopically express Wg and as a consequence induce nearby wild-type tissue to form ectopic margin bristles.

Approximately 100 mutations were obtained in our screen which cause wing margin defects in clones of mutant cells that are also homozygous for the \( f_{z2} \) loss of function mutation, \( f_{z2}^{H51} \) (see Materials and Methods). Of these, only one was associated with the formation of ectopic bristles in neighboring, wild-type wing tissue (Fig. 1B). This mutation, which we designate \( f_{z2}^{C1} \), appears to be a loss-of-function mutation in \( f_{z2} \) by the following criteria.

First, the mutation maps meiotically to a location approximately 1 centiMorgan distal to radius incompletus (ri), the expected map position given the cytological localization of the \( f_{z2} \) gene (Bhanot et al., 1996). Second, both the \( f_{z2} \) notching and ectopic bristle phenotypes associated with \( f_{z2}^{H51} f_{z2}^{C1} \) mutant cells are completely rescued when the \( f_{z2} \) coding sequence is expressed in these cells using either a Tubulin\(\alpha\)-\(f_{z2}\) transgene, which should be expressed in most or all cells, or a \( UAS-f_{z2} \) transgene driven by a \( v_{g}-\text{Gal4} \) transgene (Fig. 1C, data not shown; Materials and Methods). All of the remaining \( f_{z2} \) notch mutations obtained in the screen fully complement the \( f_{z2}^{C1} \) allele.
Fig. 1. Loss of Wg signal transduction in somatic clones of fz
\textit{H51} fz2 C1 cells in the wing. Clones of dsh73 (A) and fz
\textit{H51} fz2 C1 (B) cells cause similar wing notching phenotypes,
including the failure of mutant cells to form margin bristles (arrowheads) and the formation of ectopic margin bristles in nearby wild-type tissue (arrows). The presence of a 
\textit{Tabulin\textsubscript{AT} > f2} transgene (C) is sufficient to fully rescue the wing notching phenotypes associated with fz
\textit{H51} fz2 C1 mutant clones. Clones of fz
\textit{H51} fz2 C1 cells were induced in a ri mutant background, accounting for the gap in longitudinal vein 2; the \textgamma marker used to identify the mutant cells is not visible in these micrographs.

mutation in a fz
\textit{H51} mutant background, indicating that they are not in the fz2 gene. Third, the fz2 C1 mutation is associated with a single base change in the fz2 gene that changes codon 320 from TGG to TAG (data not shown; Materials and Methods). This creates a stop codon located at the junction between the coding sequence of the amino-terminal extracellular domain (which contains the CRD) and the remainder of the protein, which includes all seven transmembrane domains. It is unlikely that the resulting truncated protein, composed of just the extracellular domain, would retain any signal transducing activity.

As noted in the Introduction, vertebrates express a class of secreted Fz-related proteins composed of just the amino- terminal CRD-containing domain that function as endogenous antagonists of Wnt signaling. To test whether the truncated Fz2 C1 mutant protein might have a similar antagonistic function or might alter Wg signaling, we used the Gal4/UAS method to over-express Fz2 C1 protein in a stripe of cells located just anterior to the anteroposterior compartment boundary in the wing imaginal disc (Materials and Methods). Under these conditions, the Fz2 C1 protein is expressed at levels several fold above that of the endogenous wild-type protein; nevertheless, we could not detect any deviation from the normal distribution of Wg protein in the disc, nor from the normal pattern of the adult wing (data not shown). By contrast, over-expression of both the wild-type Fz2 protein as well as a GPI-linked form of the Fz2 extracellular domain under the same conditions causes enhanced accumulation of secreted Wg protein and cause gain- and loss-of-Wg signaling phenotypes, respectively (Cadigan et al., 1998; Zhang and Carthew, 1998; data not shown). Thus, we cannot observe any effect of the Fz2 C1 mutant protein, even when over-expressed, on the distribution or signaling activity of Wg, leading us to conclude that the f2 C1 mutation is an amorphic allele.

**Embryos lacking both Fz and Fz2 activity cannot transduce Wg**

To assay the possible roles of Fz and Fz2 in Wg signal transduction during embryogenesis, we generated embryos homozygous for the fz
\textit{H51} and f2 C1 mutations that derive from female germ cells that are similarly mutant for the two genes (Materials and Methods). Such embryos lack the maternal and zygotic contributions of both genes, and hence, should be devoid of Fz and Fz2 activity. We refer to these embryos below as fz- fz2- mutant embryos. To assay these embryos for Wg signal transducing activity, we examined six well defined Wg signaling events, two in the ectoderm, one in the visceral mesoderm, one in the endoderm, one in the central nervous system, and one in the somatic mesoderm. As we describe below, these embryos appear unable to transduce Wg when assayed for each event.

First, we examined the cuticular pattern formed by such double mutant embryos. The epidermis of wild-type embryos secretes a segmented cuticle, decorated on the ventral side by stereotyped bands of patterned hairs separated by broad swaths of naked cuticle (Fig. 2A). In embryos devoid of Wg activity, or of Dsh or Arm activity, most signs of segmentation are eliminated and the ventral cuticle forms a “lawn” of hairs spanning most of the anteroposterior body axis (Nüsslein-Volhard and Wieschaus, 1980; Perrimon et al., 1989; Peifer and Wieschaus, 1990; Noordermeer et al., 1994; Fig. 2B). Embryos devoid of Fz and Fz2 activity show the same characteristic “lawn” phenotype (Fig. 2C).

Second, as noted in the Introduction, the early striped expression of En in the ectoderm (Fig. 3A) is labile, unless maintained by Wg signaling from adjacent cells across the parasegment boundary. In w
\textg- dsh- and arm- mutant embryos, this expression is lost within 2 hours after the onset of gastrulation (by stage 10; DiNardo et al., 1988; Martinez Arias et al., 1988; Bejsovec and Martinez Arias, 1991; Fig. 3B). We observe a similar loss of ectodermal En expression in fz- fz2- mutant embryos (Fig. 3C).

Third, Wg signaling is essential in the visceral mesoderm for initiating a series of stereotyped constrictions that partition
the midgut (Fig. 3E). As in embryos lacking Wg, Dsh, or Arm activity (Bienz, 1994; Fig. 3F), these gut constrictions are absent in \( f^2 \) mutant embryos (Fig. 3G).

Fourth, Wg signaling is required for these Fz proteins during these experiments establish that Fz and Fz2 act upstream of Arm to transduce Wg.

Fifth, during development of the embryonic ectoderm, Wg protein moves at least a few cell diameters from secreting cells, as assayed by the accumulation of punctate dots of Wg immunoreactivity in neighboring, non-secreting cells (van den Heuvel et al., 1989; Gonzalez et al., 1991). These Eve-expressing cells are absent in \( f^2 \) mutant embryos (Fig. 3K).

Sixth and finally, during development of the somaic musculature, Eve protein is expressed in a subset of myoblasts that will give rise to the heart (Fig. 3Q) and the presence of these Eve-expressing cells is strictly dependent on Wg signaling (Wu et al., 1995; Park et al., 1996; Figs. 3R). These Eve-expressing cells are also absent in \( f^2 \) mutant embryos (Fig. 3O).

In sum, embryos devoid of both Fz and Fz2 activity appear unable to transduce Wg in any of the several developmental contexts we have examined. These results indicate an absolute requirement for these Fz proteins for Wg transduction during embryonic development.

**Distribution of Wg in \( f^2 \) mutant embryos**

During normal development of the embryonic ectoderm, Wg protein moves at least a few cell diameters from secreting cells, as assayed by the accumulation of punctate dots of Wg immunoreactivity in neighboring, non-secreting cells (van den Heuvel et al., 1989; Gonzalez et al., 1991). We therefore investigated whether the movement and apparent uptake of secreted Wg protein depends on Fz and Fz2 by examining the distribution of Wg in \( f^2 \) mutant embryos. We find that wild-type and \( f^2 \) mutant embryos show indistinguishable distributions of punctate Wg staining during the first two hours following germ band extension (data not shown), consistent with the view that neither Fz nor Fz2 protein are required for the movement of secreted Wg during this phase of development. However, the \( f^2 \) mutation is expected to generate a protein which is truncated after the sixth transmembrane domain (Jones et al., 1996). Hence, if this protein is stable and reaches the cell membrane, it might be able to bind and regulate the movement of secreted Wg even though it can no longer transduce Wg signal. Wg expression dissipates in \( f^2 \) mutant embryos shortly after this early stage, as expected given the loss of En expression in neighboring cells across the AP compartment boundary, preventing us from examining later aspects of Wg movement in these embryos.

**Fz and Fz2 transduce Wg via the regulation of Armadillo**

Most, if not all, Wg signal transducing events involve the modification and up-regulation of Armadillo (Arm) protein (Riggleman et al., 1990; Peifer et al., 1994; Willert and Nusse, 1998). We therefore performed two experiments to test whether Fz and Fz2 transduce Wg through the regulation of Arm. These experiments establish that Fz and Fz2 act upstream of Arm to transduce Wg.

In the first experiment, we assayed Arm expression in \( f^2 \) mutant embryos. In wild-type embryos, Wg signaling is associated with stabilization of Arm protein and its consequent accumulation in a distinctive pattern of segmental stripes, each straddling a stripe of Wg-expressing cells (Riggleman et al., 1990; Peifer et al., 1999; Fig. 4A). This up-regulation is not observed in \( wg \) embryos (data not shown; Fig. 4B).

In the second experiment, we asked whether expression of a truncated, constitutively active form of Arm, \( \Delta \text{Arm} \) (Zecca et al., 1996), could drive the Wg signal transduction pathway in

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**Fig. 2.** Cuticular phenotype of \( f^H \) mutant embryos. Wild-type embryos (A) form a segmented larval cuticle decorated with bands of thick ventral hairs separated by naked cuticle, whereas \( wg^* \) (B) and \( f^H \) mutant embryos (C) form abnormally short, unsegmented larval cuticles that bear a continuous "lawn" of ventral hairs. \( f^H \) mutant embryos in which a constitutively active form of Arm, \( \Delta \text{Arm} \), is expressed in alternating segmental primordia using the Gal4/UAS method, form corresponding stripes of naked cuticle (D), placing Fz and Fz2 upstream of Arm in the Wg signal transduction pathway. In contrast, expression of Wg instead of \( \Delta \text{Arm} \) in alternating stripes in \( f^H \) mutant embryos (E) does not rescue the lawn phenotype. Expression of Wg in alternating stripes in an otherwise wild-type embryo (F) suppresses the normal formation of ventral hairs (compare with A) confirming that the Gal4/UAS method generates ectopic Wg signaling.
In this experiment, ∆Arm was expressed with the UAS/Gal4 method using a hairy-Gal4 driver line which is active in alternating segmental primordia (Brand and Perrimon, 1993). As shown in Fig. 2D, expression of ∆Arm in alternating segmental stripes in fz- fZ2- mutant embryos causes them to form corresponding stripes of naked cuticle. This result indicates that the constitutive activity of ∆Arm bypasses the normal requirements for Fz and Fz2 in activating the Wg transduction pathway.

As a control for the second experiment, we tested the consequences of over-expressing Wg instead of ∆Arm in fz- fZ2- mutant embryos. As shown in Fig. 2E, expression of a UAS-wg transgene (Zecca et al., 1996) under the control of the hairy-Gal4 driver line failed to rescue the fz- fZ2- mutant phenotype, even though hairy-Gal4 driven expression of the same transgene in otherwise wild-type embryos causes ectopic Wg signal transduction (Fig. 2F; Wilder and Perrimon, 1995). Thus, ventral ectodermal cells of fz- fZ2- mutant embryos appear unable to transduce Wg, even when it is ectopically expressed.

**Presumptive wing cells lacking both Fz and Fz2 activity cannot transduce Wg**

Wg signaling plays a variety of roles during development of the imaginal discs. Here, we focus on the roles of Fz and Fz2 in the wing disc, and particularly in the wing pouch which gives rise to the adult wing blade. As noted above, Wg is expressed in the wing pouch of late third instar discs in a thin stripe of cells straddling the interface between the dorsal and ventral compartments. Wg emanating from this stripe acts at
Wingless transduction by the Frizzled proteins

in wing discs bearing “twin spots” formed by sibling fz\textsuperscript{HS1} fz\textsuperscript{2C1}fz\textsuperscript{HS1} fz\textsuperscript{2C1} and +/+ clones which arise following mitotic recombination in heterozygous fz\textsuperscript{HS1} fz\textsuperscript{2C1} mother cells. In this experiment, the fz\textsuperscript{HS1} fz\textsuperscript{2C1} mutant clones are marked by the absence of expression of Green Fluorescent Protein (GFP), while their sibling, wild-type clones are marked by enhanced expression of GFP (Materials and Methods).

When mitotic recombination is induced early in development, during the first larval instar, twin spots are found in most portions of the mature wing disc (Fig. 6B). However, they are not found in the wing pouch, which is destined to form the wing blade. Here, we find only wild-type clones (Fig. 5B), indicating that their fz\textsuperscript{HS1} fz\textsuperscript{2C1} twins have been lost from the epithelium.

When mitotic recombination is induced later in development, such as the beginning of the third instar, wild-type clones within the wing pouch are sometimes accompanied by a mutant twin spot, although the mutant spot is typically smaller and shows abnormally low or undetectable levels of Vg and Dll expression (Fig. 5C). When mitotic recombination is induced at later times during the third larval instar, wild-type clones are usually accompanied by their mutant twins, which express both Dll and Vg.

We also generated fz\textsuperscript{HS1} fz\textsuperscript{2C1} mutant clones during embryogenesis using the Minute technique (Morata and Ripoll, 1975) to give these cells a growth advantage relative to the surrounding wild-type tissue. Such clones can fill large portions of either the anterior (A) or posterior (P) compartment of the entire disc, except for the wing pouch from which they appear to be excluded (data not shown).

We interpret these results as evidence that Wg signal transduction is abolished in presumptive wing cells lacking both Fz and Fz2 activity. As a consequence, cells that lack both activities cannot proliferate normally and are lost from the epithelium. We attribute the ability of small, late-induced clones of fz\textsuperscript{HS1} fz\textsuperscript{2C1} cells to survive and express Vg and Dll to the transient perdurance of Fz and/or Fz2 proteins in the mutant cells.

We note that although fz\textsuperscript{HS1} fz\textsuperscript{2C1} clones survive and contribute to regions of the wing disc other than the wing pouch, their behavior in these regions appears abnormal. For example, in the prospective wing hinge region they are typically smaller than their wild-type twins, round in shape, and have smooth borders. In addition, when such hinge clones are positioned close to the D/V boundary, they express Vg, but not Dll, and the same is true of large fz\textsuperscript{HS1} fz\textsuperscript{2C1} clones generated using the Minute technique (Fig. 5B and data not shown). In this respect the mutant cells resemble wild-type cells located more proximally along the DV boundary, which express Vg in response to the activation of Notch, but do not express Dll. Wg signaling is required for the control of growth and pattern in portions of the wing disc other than the wing pouch (Neumann and Cohen, 1996; Ng et al., 1996), and we suggest that the abnormal behavior of fz\textsuperscript{HS1} fz\textsuperscript{2C1} clones in these regions reflects their failure to transduce Wg.

Requirements for Fz and Fz2 activity in the mesonotum

The wing imaginal discs also give rise to the fuselage of the adult second thoracic segment, the mesonotum, the anterior dorsal surface of which is decorated with a stereotyped pattern
of large bristles (Fig. 7A). Wg is expressed in a longitudinal stripe in the developing half-mesonotum derived from each wing disc and this stripe is positioned just lateral to a line of four large bristles (Phillips and Whittle, 1993). These are the anterior and posterior dorsocentral bristles and the anterior and posterior scutellar bristles. The formation of these bristles has been reported to be Wg dependent (Phillips and Whittle, 1993).

We find that clones of *fz* H51 *fz2 C1* cells generated using the Minute technique during embryogenesis can fill the entire mesonotum of the adult fly although, as described above, they fail to contribute to the wing blade. The bristles of such mutant mesonota show the characteristic frizzled polarity phenotype. However, the pattern is otherwise normal, including the presence of both dorsocentral bristles and both scutellar bristles at their stereotyped positions in each half (left and right) of the mesonotum (Fig. 7C). To assay the Wg-dependence of these bristles, we performed a similar experiment, this time using the Minute technique to generate large clones of *wg* cells. In this case, clones of mutant cells that filled the mesonotum typically formed only a single dorsocentral bristle at a position approximately equidistant between the positions where the two dorsocentral bristles normally form (Fig. 7B). Similarly, they tended to form only one scutellar bristle at a site between the normal positions of the anterior and posterior scutellar bristles.

The different bristle pattern phenotypes associated with *wg* and *fz* H51 *fz2 C1* clones could, in principle, reflect different degrees of perdurance, or cell autonomy, associated with the two mutant conditions. However, an alternative possibility is that Wg might be transduced in this context by a receptor other than Fz or Fz2.

**Redundant roles of Fz and Fz2 in transducing Wg throughout development**

Animals homozygous for null mutations of *fz* survive to adulthood and show little if any evidence that they are compromised for Wg signal transduction. To determine whether animals homozygous for the *fz2 C1* mutation are compromised for Wg signal transduction, we recombined this mutation away from the *fz H51* mutation and then assayed the fate of *fz2 C1* homozygotes derived from heterozygous *fz2 C1/*+ parents. We find that such homozygotes can develop into normal first instar larvae which in turn can give rise to adults, although they tend to be developmentally delayed relative to their heterozygous siblings and give rise to abnormally small, sterile males and females (see Materials and Methods). Nevertheless, surviving *fz2 C1* homozygous flies are normally proportioned and patterned, and lack any overt phenotypes associated with reduced Wg signaling (data not shown). Thus, it appears that zygotic activity of the *fz* gene is not essential for Wg signal transduction, provided that a wild-type allele of *fz* is present.

To examine further the functional redundancy between *fz* and *fz2*, we performed the following three experiments. First, we fertilized eggs derived from homozygous *fz H51 f2 C1* germ cells with sperm from heterozygous *fz2 C1/*+ or *fz H51/*+ males. The resulting *fz2 C1/*fz H51 f2 C1* or *fz H51/*fz H51 f2 C1* progeny lack...
any functional Fz or Fz2 protein derived from maternal gene expression, but do express functional Fz or Fz2 protein, depending on which wild-type gene is introduced paternally. In both cases, the embryos appear to develop normally (e.g. Fig. 6D,F) and form phenotypically wild-type larvae (data not shown), indicating that either protein can transduce most or all Wg signaling events during embryogenesis.

The second experiment we performed was to generate homozygous fz2C1 embryos from eggs derived from homozygous fz2C1 germ cells, and then test these embryos for their ability to transduce Wg using the six assays described above for analyzing Wg signaling in fzH51 fz2C1 mutant embryos. Although these fz2C1 mutant embryos should be devoid of functional Fz2 protein, we find that they are indistinguishable from wild-type embryos in their ability to transduce Wg by all six assays (Fig. 3D,H,L,P,T; data not shown). In addition, they show the normal increase in Arm abundance in response to Wg signaling (data not shown).

Third, we examined the behavior of clones of fz2C1 cells in the wing imaginal disc. In experiments similar to those performed for the fzH51 fz2C1 genotype, we find that the fz2C1 clones and their wild-type sibling clones are found throughout the wing disc, including the wing pouch, and are of similar size and appearance irrespective of the time they were induced (Fig. 5D). When the Minute technique was used, the resulting fz2C1 clones filled large portions of either the A or P compartment, including the entire A or P portion of the wing pouch (data not shown). In both cases, the mutant clones show normal expression of Dll and Vg, and contributed to phenotypically normal adult wings. Indeed, by inducing a high frequency of fz2C1 clones early in development using the Minute technique, we were able to generate flies in which virtually all cells in the thorax and head are mutant (the Minute technique is not effective in the abdomen, which remains mosaic). These flies appeared phenotypically normal in all respects (data not shown).

Thus, we conclude that Fz and Fz2 proteins are functionally redundant, with either protein being able to bear the full burden of Wg signal transduction in most, if not all, contexts throughout development.

DISCUSSION

A large body of circumstantial evidence has implicated Fz proteins as Wnt receptors during animal development (see Introduction). However, the consequences of eliminating the
endogenous activities of these proteins have been studied in relatively few cases, and the results so far have failed to establish an absolute requirement for Fz proteins in Wnt signal transduction. In *Drosophila*, most Wnt signaling events involve a single Wnt, Wg (D-Wnt1), and two Fz proteins, Fz and Fz2, which have been proposed as candidate receptors. However, null mutations of the *fz* gene do not cause phenotypes that are related to reductions or loss of Wg signaling activity. Moreover, loss-of-function mutations have not been reported for the *fz2* gene, making it difficult to assess the requirement for Fz2 in Wg signal transduction, either alone or in conjunction with Fz. As a consequence, considerable uncertainty remains about the normal roles of these proteins in Wg reception.

Here, we report the isolation of a likely null allele of *Drosophila fz2*. Using this mutation together with an amorphic mutation in *fz*, we find an absolute requirement for Fz proteins in mediating most responses to Wg signaling during development. We also show that Fz and Fz2 are functionally redundant with respect to Wg signaling: the presence of either protein appears sufficient to transduce most if not all Wg signals throughout development. Finally, we find that Fz and Fz2 function upstream of Arm, a protein which acts within cells to transduce Wg. Taken together with previous evidence that Fz and Fz2 have Wg binding activities (Bhanot et al., 1996; Nusse et al., 1997; Cadigan et al., 1998), our results argue that the two proteins constitute the primary Wg receptors in *Drosophila*. These results have several implications which we consider below.

**Evidence against subsets of Wg receptors which mediate distinct Wg responses**

Previous studies of *wg* mutations have raised the possibility that different receptors mediate distinct Wg outputs, perhaps through direct interactions with different functional domains in Wg protein, or in different developmental contexts. For example, some partial loss-of-function mutations in *wg* have been reported to block the specification of “naked” ventral cuticle between each band of ventral hairs secreted by the ventral ectoderm of the embryo, whereas others alter only the pattern of distinct hair types within each band (reviewed by Dierick and Bejoovec, 1999). These different responses have led to the proposal that each output reflects transduction of Wg by a different receptor (Hays et al., 1997). Similarly, Fz itself has been proposed to play a role in Wg signal transduction during embryogenesis, but not during the development of the imaginal discs where Fz2 has been proposed to be the primary Wg receptor (Kennerdell and Carthew, 1998; Zhang and Carthew, 1998). Our evidence contradicts these proposals by showing that Fz and Fz2 are each capable of mediating virtually all Wg responses during normal development.

**Evidence against other Wg receptors**

Circumstantial evidence has also been presented in support of candidate Wg receptors other than Fz and Fz2. In particular, loss-of-function mutations in the transmembrane receptor Notch have been shown to mimic many of the phenotypes associated with reduction or loss of Wg signaling, consistent with a receptor-ligand relationship (Couso and Martinez Arias, 1994). In addition, at least two other Fz-like proteins have been identified in *Drosophila* (M. Boutros and M. Mlodzik; A. Sato and K. Saigo, as cited by Bhat, 1998), raising the possibility that these other proteins can transduce Wg in at least some contexts. However, the complete absence of detectable Wg signal transduction in cells devoid of Fz and Fz2 activity in virtually all of the contexts we have assayed suggests that these other proteins are normally not capable of transducing Wg when both Fz and Fz2 are absent. Hence, we suggest that if these, or other, proteins have a role in Wg reception, it would be through the modulation of Fz and Fz2 activities. For example, they could facilitate or antagonize interactions between Wg and Fz proteins on the outside of the cell, or they could modulate interactions between Fz proteins and their down-stream effectors on the inside of cell.

We note that our results do not rule out the possibility that additional Wg receptors will exist and play significant roles in at least some developmental contexts. One possible case is suggested by our finding that clones of *fz*<sup>H51</sup> *fz2<sup>C1</sup> cells which populate the mesonotum form the normal pattern of dorsocentral and scutellar bristles, a response which appears to be at least partially dependent on Wg signaling (Phillips and Whittle, 1993). Hence, Wg-dependent patterning of these bristles may depend on additional Wg receptors other than Fz and Fz2. However, our results suggest that such examples will be relatively rare exceptions to the general role of Fz and Fz2 as Wg receptors. Our results also leave open the possibility that Fz and Fz2 function in the context of a larger receptor complex that includes other components which are similarly essential for binding and transducing Wg.

**Potential roles of Fz proteins in generating and interpreting gradients of secreted Wg**

The distribution of Fz2 protein is generally complementary to that of Wg itself, peaking in cells far from the source of secreted Wg, but expressed at low levels in cells close to the source (Cadigan et al., 1998). This, and related observations on the effects of over-expression of Fz2, have led to the proposal that the distribution of Fz2 plays a significant role in modulating the spread and accumulation of Wg, once secreted (Cadigan et al., 1998). However, our finding that clones of *fz2<sup>C1</sup>* cells in the wing disc, and indeed, entirely *fz2<sup>C1</sup>* mutant wing discs, can give rise to phenotypically normal wings challenges this proposal. It is possible that the Fz2<sup>C1</sup> mutant protein, which is composed of just the N-terminal extracellular domain, might retain the ability to modulate the movement and accumulation of Wg protein, even though it lacks transducing activity. However, we think this unlikely because we fail to detect any effect of over-expression of the mutant protein on either Wg signaling or the distribution of Wg protein during wing development. Hence, the ability of *fz2<sup>C1</sup>* mutant wing discs to develop normally can be interpreted as evidence that Fz2 is not required to modulate the spread or accumulation of extracellular Wg.

Wg appears to function as a gradient morphogen during imaginal disc development, inducing discrete outputs in terms of gene expression and pattern as a function of its concentration (Zecca et al., 1996; Neumann and Cohen, 1997; Cadigan et al., 1998). Hence, the ability of animals lacking either zygotic Fz or Fz2 activity to develop into normally patterned flies suggests that both proteins can transduce the same concentration of Wg with similar effectiveness, yielding the same outputs for a given input concentration. It is possible that Fz or Fz2 have similar, innate capacities for binding and transducing Wg. Alternatively,
their abilities to bind and transduce Wg may depend on other proteins that facilitate these interactions, allowing them to interpret a given Wg gradient in the same way.

**Planar polarity and the mode of action of Fz**

Most epidermal cells of the adult fly differentiate structures such as hairs or bristles, which derive from polarized extensions of their cytoskeletons. In general, these structures have a common orientation, all pointing in the same direction, a phenomenon referred to as planar polarity (Nubler-Jung, 1987). Planar polarity is altered in cells lacking Fz activity, presumably because the mutant cells are unable to respond properly to an as yet unknown polarizing signal.

Our evidence that Fz is one of two primary Wg receptors has implications for its role in planar cell polarity. The most straightforward of these is the possibility that the polarizing signal is a Wnt which can also bind to the Wg binding site of Fz, but once bound, induces an intracellular response that is distinct from that elicited by Wg. Curiously, previous studies have implicated the same cytosolic protein, Dsh, in transducing both the polarity signal and the conventional Wg signal (Theisen et al., 1994; Neumann and Cohen, 1997; Tomlinson et al., 1997; Axelrod et al., 1998). Our present findings focus further attention on the relationship between Dsh and transduction of both Wg and the putative polarizing signal because they indicate that Fz alone is capable of transducing both outputs through the agency of Dsh, each in response to a different ligand.

The dual roles of Fz and Dsh in transducing both Wg and the polarizing signal pose the question of how distinct ligands can activate different transduction pathways through their actions on the same receptor. It is possible that Fz activity is modulated by accessory proteins which influence which ligand it will bind, and what transduction pathway it will activate in response to ligand. For example, the structurally related, serpentine calcitonin-receptor-like receptor (CCLR) has recently been shown to bind either of two ligands depending on the presence of specific receptor-activating-modifying proteins (RAMPs) (McLatchie et al., 1998). A similar mechanism might account for how Fz can transduce both Wg and the polarizing signal through the activation of distinct intracellular pathways even in the same cell.

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**REFERENCES**


Bhat, K. M. (1998). frizzled and frizzled 2 play a partially redundant role in wingless signaling and have similar requirements to wingless in neurogenesis. *Cell* 95, 1027-1036.


**Wingless transduction by the Frizzled proteins** 5451


