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Overexpressing the Drosophila frizzled 2 gene affects the wingless pathway

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OVEREXPRESSING THE DROSOPHILA FRIZZLED 2 GENE AFFECTS THE WINGLESS PATHWAY

A Thesis

Presented to

The Faculty of the Department of Biological Sciences

In Partial Fulfillment

Of the Requirements for the Degree

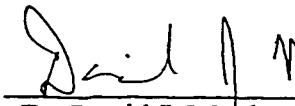
Master of Sciences

by

Matthew Palmer Fish

May 1999

APPROVED FOR THE DEPARTMENT OF BIOLOGICAL SCIENCES



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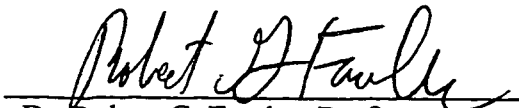

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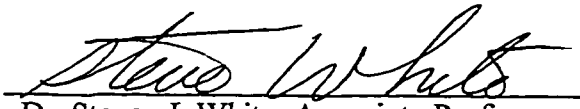

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ABSTRACT

OVEREXPRESSING THE DROSOPHILA FRIZZLED 2 GENE
AFFECTS THE WINGLESS PATHWAY

by Matthew Palmer Fish

Wingless (Wg) signaling in *Drosophila* is essential for normal development during several distinct stages. Although many of the components involved in Wg signaling have been determined, a candidate receptor for the Wg ligand has only recently been identified. The *Drosophila Frizzled 2* (*Dfz2*) gene has been shown to function as a Wg receptor in cell culture. Transgenic *Drosophila* lines have been created that express two different forms of *Dfz2*. This study shows that a truncated version of Dfz2 consisting of only the extracellular ligand binding domain bound to the cell membrane can inhibit Wg signaling *in vivo*. In addition, it is also shown that full length Dfz2 can function as a Wg receptor by inducing target genes and creating Wg overexpression phenotypes *in vivo*. We show that Dfz2 can stabilize Wg far from its source in wing discs. Further, small deficiencies have been created by X-ray mutagenesis that abolish *Dfz2*.

ACKNOWLEDGMENTS

This work could not have been completed without the support of friends and family. I would like to thank David Matthes for supporting this work, for teaching me how to evaluate science in a critical manner, and for suggestions on this thesis. I thank Roel Nusse for allowing me to perform this work in his lab and for providing an environment in which to learn. I also thank Steven White and Robert Fowler for their critical comments on this thesis. Ken Cadigan, Eric Rulifson, Cindy Harryman-Samos, Marcel Brink, Matt Scott, and Derek Lessing have provided instruction, support, and reagents that made this work possible. I am indebted to all of the members of the Nusse and Scott labs that I have had the distinguished opportunity to work with. Finally, I thank Margaret Ann Fish, Raymond George Fish, and Gregory Shannon Fish for their love and support.

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Introduction

The *Wnt* genes represent a family of genes that encode secreted glycoproteins, are involved in cell fate determination and are essential for normal development in many different animals. For instance, in the mouse, expression of the *Wnt-1* gene is seen between 8 and 14 days of embryogenesis in the developing central nervous system (McMahon and Bradley, 1990). A naturally occurring mutation in *Wnt-1* results in the *swaying* phenotype (Thomas et al., 1991). This phenotype is due to loss of the cerebellum and a large portion of the midbrain during neural development. Conversely, retroviral insertion mutations that activate *Wnt-1* are found in mouse mammary tumors (Nusse and Varmus, 1992). In this case overexpression of a *Wnt* gene leads to uncontrolled cell proliferation in mammary tissue. In *Xenopus* embryos, injection of mouse *Wnt-1* mRNA induces axis duplication of the head (McMahon and Moon, 1989). In addition, several other members of the *Wnt* family can produce axis duplication when injected into individual blastomeres of frog embryos. These results suggest that *Wnt* genes are involved in the induction of dorsal mesoderm and the establishment of the body axis in *Xenopus*.

The *wingless* gene (*wg*), in the fruit fly *Drosophila melanogaster*, is a member of the *Wnt* family, and the *Wnt-1* ortholog (Rijsewijk et al., 1987). *wg* plays a crucial role during several periods of *Drosophila* development. Previous work on *wg* has shown that it is involved in signal transduction between adjacent rows of cells in the developing embryo epidermis and in imaginal disc development (Klingensmith and Nusse; 1994; Neumann and Cohen, 1997). In the wing disc Wg is thought to act as a morphogen

(Zecca, et al., 1996). Morphogens are classified as proteins that diffuse from a central point and effect target cells differentially depending on their distance from the source and the concentration of the morphogen.

In the first few hours of development the *Drosophila* embryo is divided into compartments called parasegments which are subdivisions of what ultimately becomes a body segment. Initially, parasegments are defined by the expression pattern of several segment polarity genes including *wg* itself (Noordermeer et al., 1995). As development progresses, patterns of denticles (cuticular protrusions resembling short stout hairs) begin to form on the ventral side of the embryo which also define the parasegments. These denticles are formed in the anterior portion of the parasegments (Figure 1A). Mutations in *wg* result in cells that are unable to signal to adjacent cells. In these embryos, a continuous body of denticles is formed (Figure 1B) that obscures parasegmental and segmental boundaries (reviewed by Perrimon, 1994) and is classified as a segment polarity phenotype. Conversely, overexpressing Wg leads to a "naked" cuticle phenotype (Noordermeer et al., 1992) where these denticles are lost (Figure 1C). In leg discs, the larval progenitors of adult structures, *wg* is required for dorsoventral and anteroposterior axial patterning (Wilder and Perrimon, 1995) (Figure 1E), while in the wing *wg* is necessary for growth of the wing blade as well as bristle patterning at the margin (Couso et al., 1994) (Figure 1D). In leg discs, loss of *wg* results in truncation of leg structures and in the wing disc loss of *wg* results in the absence of wings, the phenotype from which this gene derived its name.

Figure 1. Wg Expression Affects Embryonic and Imaginal Disc Development.

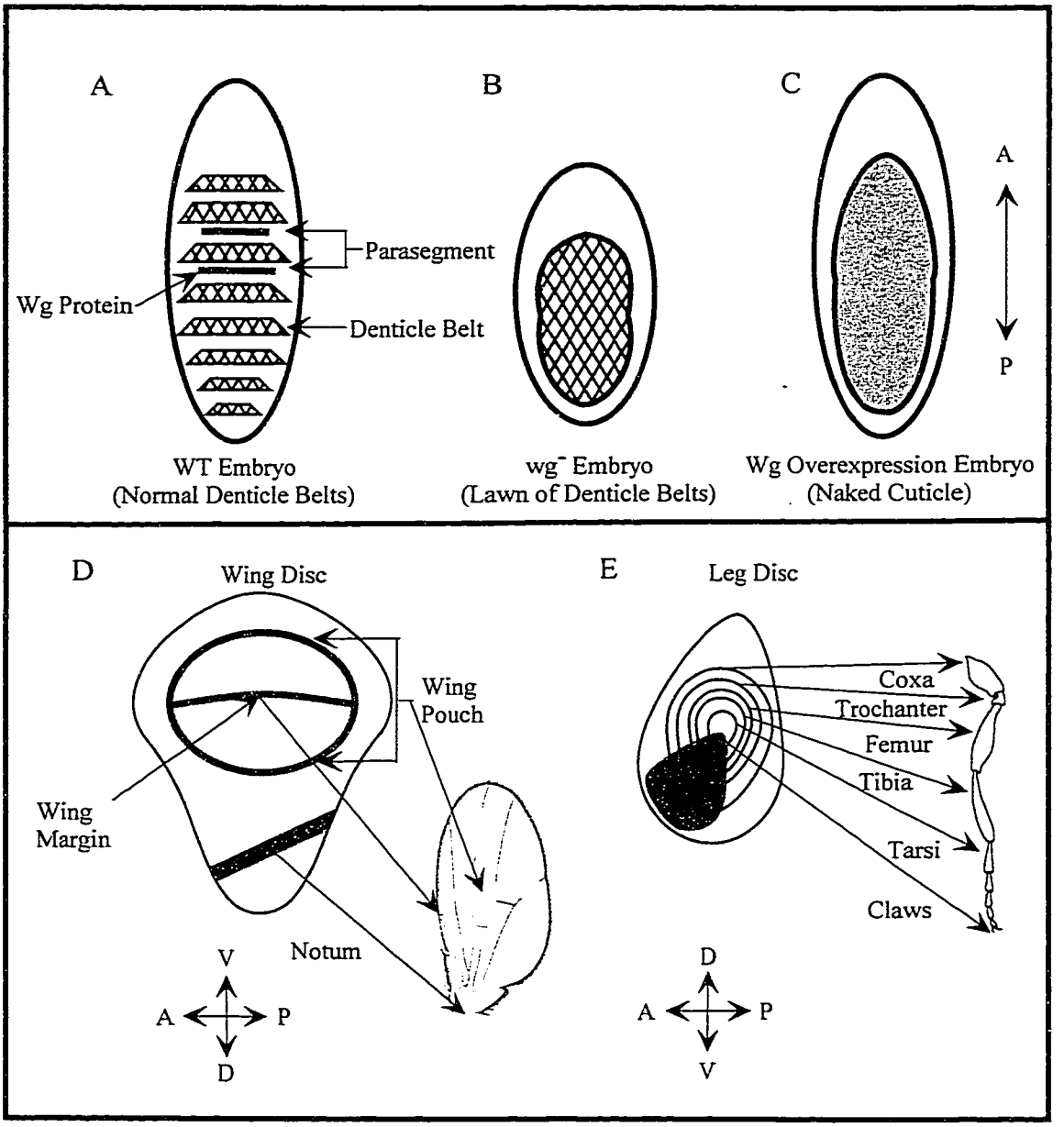

Figure 1. Wg Expression Affects Embryonic and Imaginal Disc Development.

In wild type embryos Wg is seen in the posterior portion of each parasegment. The normal array of denticle belts on the ventral side of the embryo (A) expand and obscure parasegmental boundaries in *wg* mutants (B). Overexpression of Wg inhibits denticle formation and only naked cuticle is seen (C).

wg is also required for the normal development of imaginal discs. In the wing disc (D) Wg is expressed around the wing pouch and in a stripe of cells that represent the entire wing margin. The notum area also expresses Wg. In the leg disc (E) Wg is expressed in a wedge of cells in the ventral and anterior portion of the leg. Third instar imaginal discs are shown, Wg protein is in green, and adult appendages are shown to the right for reference.

Wg signaling is a multi-step process that involves several different gene products. Most of the effort to define the components of the *wg* pathway has been done in parasegments of the embryonic epidermis. This work has been accomplished mainly by genetic screens. From this work a model for Wg signaling in the embryonic epidermis has been proposed (Cadigan and Nusse, 1997) (Figure 2). In the embryonic epidermis, the Wg signal is transduced through several other gene products and ultimately induces the expression of the gene *engrailed* (*en*) in cells posterior to the Wg secreting cells (Noordermeer et al., 1992). The product of *en* is essential for the formation of naked cuticle. In this model of the *wg* pathway, the *porcupine* gene product is necessary for the transport and secretion of Wg protein at the cell surface enabling it to signal to adjacent cells. When Wg binds to its receptor from the Frizzled family, the phosphoprotein Dishevelled (Dsh) is recruited to the cell membrane (Axelrod et al., 1998). Dsh represses the activity of the kinase Zeste-white 3 (Zw3) (Diaz-Benjumea and Cohen, 1994) which in conjunction with Axin and Adenomatous Polyposis Coli (APC) normally destabilize Armadillo (Arm) the beta-Catenin homolog (Ahmed et al., 1998). Thus, upon signaling, Arm is stabilized and can enter the nucleus where along with T-Cell Factor (TCF) it is able to promote expression of target genes including En (Figure 3).

To understand the events involved in Wingless signaling, the full repertoire of molecules involved must be characterized. Although many of the components of Wg signaling have been determined, candidate receptors for the Wg ligand have long remained elusive. None of the products downstream of Wg that have been identified so

Figure 2. Wg Regulation in the Embryonic Epidermis.

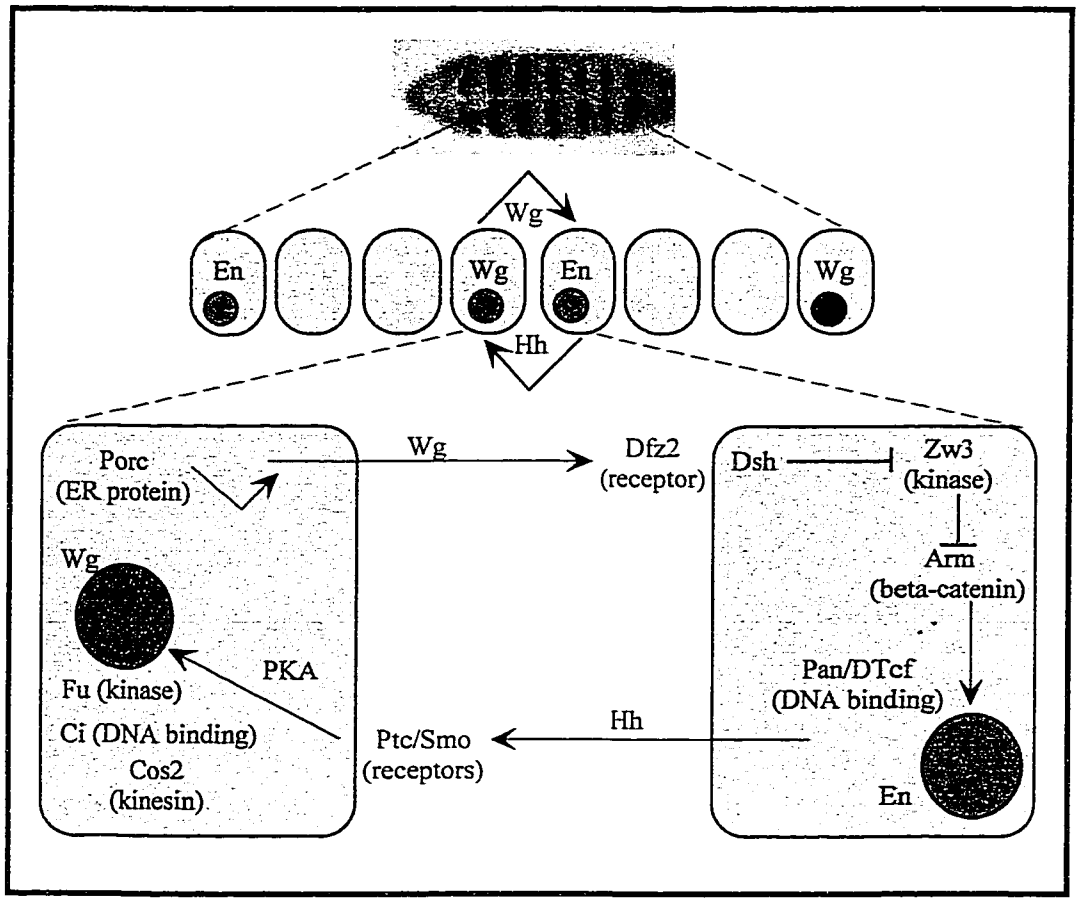

Figure 2. Wg Regulation in the Embryonic Epidermis.

Wg secretion is responsible for activating target genes in neighboring cells and acts to establish parasegmental boundaries. Wg expression (dark blue) and Engrailed (En) expression (brown) are shown in a *Drosophila* embryo (top panel, anterior is to the left). The center panel shows two parasegments with Wg expression in the posterior and En expression in the anterior of each parasegment. Signaling by Wg is necessary to maintain En expression in adjacent cells (bottom panel, see text). Figure from Cadigan and Nusse (1997).

Figure 3. Components of the Wnt/Wg Pathway.

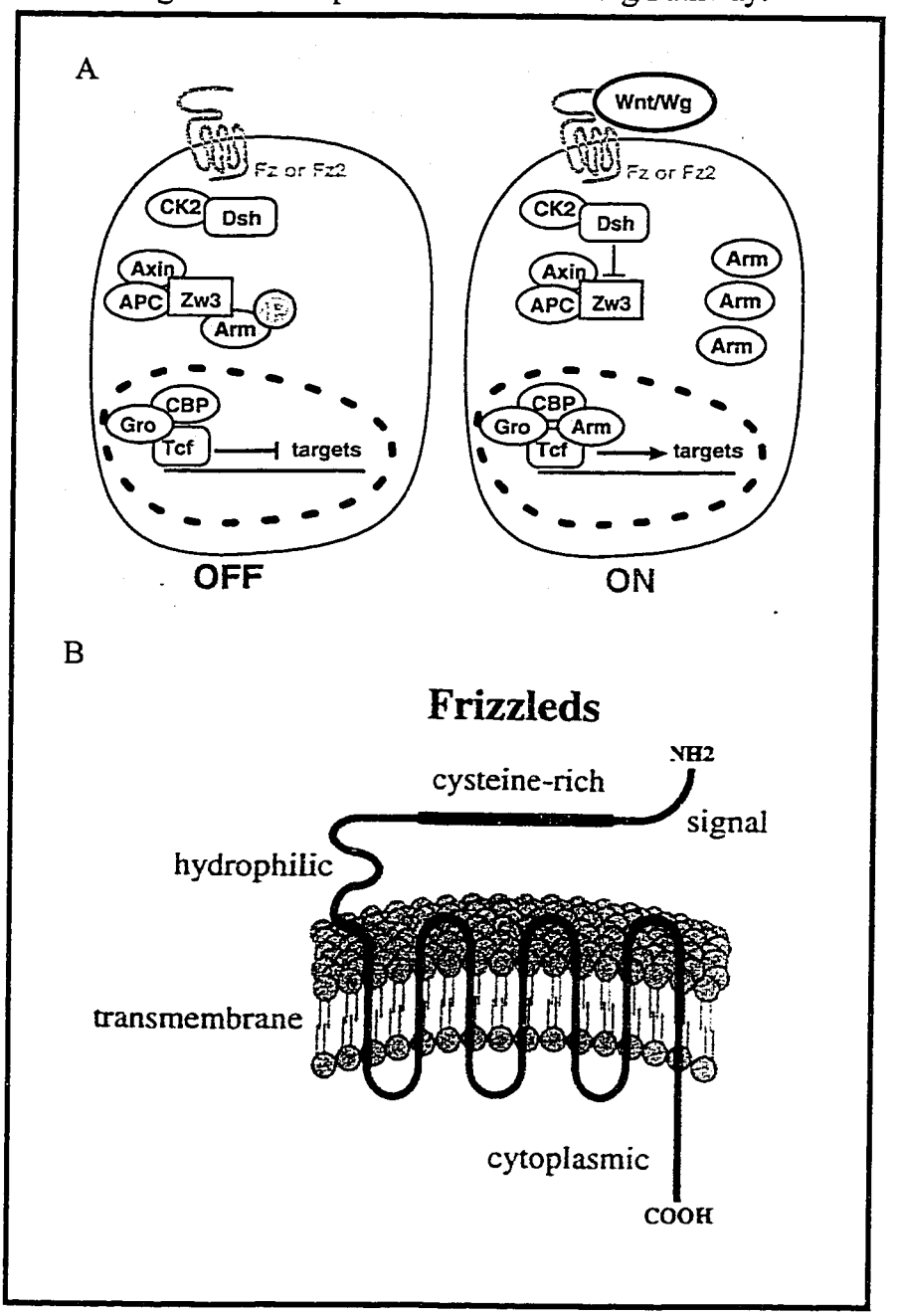

Figure 3. Components of the Wnt/Wg Pathway.

In the absence of a Wnt/Wg signal, Armadillo (Arm), which has been shown to associate, perhaps in a complex, with the proteins Axin, APC, and Zw3, is destabilized by the kinase Zeste-white 3 (Zw3) in the cytoplasm and can be degraded. Upon signaling, a Wnt/Wg interacts with its receptor from the Frizzled family of seven pass transmembrane proteins. This interaction results in Dishevelled (Dsh) inactivating Zw3 and allows dephosphorylated Arm to accumulate in the cytoplasm. This form of Arm is then able to enter the nucleus where along with the transcription factor Tcf it can inhibit the repressive activity of Groucho (Gro) to initiate target gene expression.

The structural elements of Frizzled proteins (B) include a signal sequence followed by a cysteine rich domain (CRD) in which10 invariantly spaced cysteines are found outside of the cell membrane. A hydrophilic region links the CRD to a seven transmembrane motif. The C-terminus is located in the cytoplasm. Panel B is from Wodarz and Nusse (1998).

far are considered to be the Wg receptor, as judged by sequence data (Bhanot et al., 1996). However, recent evidence shows that the *Drosophila frizzled 2* (*Dfz2*) gene can transduce the Wg signal in cell culture (Bhanot et al., 1996). This conclusion was made based on experiments showing that cells normally unresponsive to Wg gained the ability to stabilize the Armadillo protein (a product downstream of Wg in the pathway) when transfected with a Dfz2 expression construct (Figure 4). In addition, the ability of cells expressing a truncated version of Dfz2 to bind Wg was evaluated. In this assay the extracellular domain of Dfz2, that is a cysteine-rich domain (CRD) proposed to constitute the ligand binding-domain, was expressed on the cell surface as a glycosylphosphotidylinositol (GPI)-anchored protein. Incubation of these cells with Wg protein followed by anti-Wg antibodies showed strong surface staining and indicated that the CRD domain is at least a significant part of the Wg ligand-binding domain (Bhanot et al., 1996). In these experiments Arm was not stabilized suggesting that the CRD of Dfz2 could bind Wg in a nonproductive manner.

The *Dfz2* gene is located at 76A on polytene chromosomes and encodes a seven pass transmembrane protein (Figure 3B) which has homology to Smoothened (Smo) and Frizzled (Fz) in *Drosophila*. Smoothened has been shown to interact with Patched in Hedgehog signaling (reviewed in Johnson and Scott, 1997) and Frizzled is involved in tissue polarity (the orientation of ommatidia in the eye and bristles in the wing) (Krasnow and Adler, 1994; Zheng et al., 1995). Although Frizzled can bind Wg in cell culture, it does not seem to be involved in Wg signaling *in vivo* based on the evidence that null

Figure 4. Dfz2 can Confer Wg Responsiveness in S2 Cells.

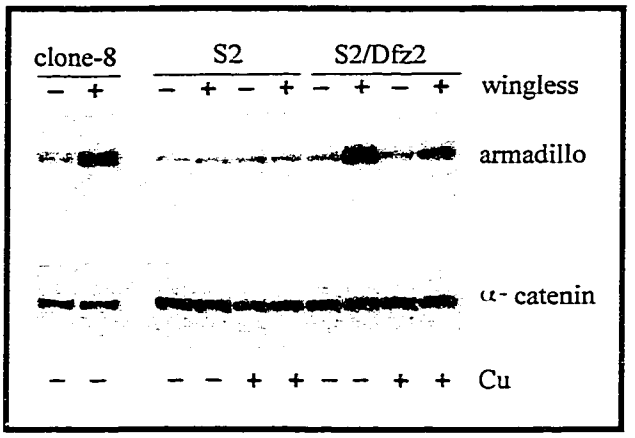

Figure 4. Dfz2 can Confer Wg Responsiveness in S2 Cells.

Clone 8 cells normally express Dfz2 and can accumulate Armadillo (Arm) upon incubation with Wg (Lane B) S2 cells which normally do not express Dfz2 or respond to Wg (Lanes C-F) can be induced to accumulate Arm to high levels when transfected with Dfz2 (Lanes G-I). Cu is used to induce transgene expression under the metallothioneine promoter and alpha-catenin is shown as a loading control. Figure from Bhanot et al., (1996).

mutations of the gene do not have *wg* like phenotypes (Bhanot et al., 1996; Krasnow and Adler, 1994).

Despite the strong evidence that Dfz2 binds the Wg ligand and transduces signaling in cell culture experiments, the role of Dfz2 *in vivo* has yet to be described. This task is made difficult as there are currently no *Dfz2* mutants available with which to evaluate its loss of function phenotype and compare to *wg* phenotypes. To address these issues transgenic *Drosophila melanogaster* lines have been created that express either the full length *Dfz2* gene or a truncated version consisting of the CRD linked to a GPI anchor. Overexpression of these transgenes was targeted (Brand and Perrimon, 1993) to the developing embryo as well as imaginal discs and their subsequent adult structures by using tissue- and stage-specific Gal4 enhancer lines (see Experimental Procedures). Phenotypes reminiscent of those in *wg* mutants were observed when the Dfz2-GPI construct was overexpressed, suggesting that it acts as a dominant negative for Wg signaling. When the full length Dfz2 construct was overexpressed the phenotypes observed were similar to Wg overexpression phenotypes. It was also observed that both Dfz2 and Dfz2-GPI can stabilize Wg far away from its source of expression in wing imaginal discs.

In addition fly lines were made mutant at the *Dfz2* locus by X-ray mutagenesis. Several lines were found that lack *Dfz2* and are embryonic lethal. Some of these lines are small deficiencies that will be useful for further characterization of the role of Dfz2 in Wg signaling and in creating additional alleles of *Dfz2*.

Results

Dfz2-GPI Blocks Wg Signaling

Previous work has shown that the cysteine rich domain (CRD) of Dfz2 is sufficient to bind the Wg ligand (Bhanot et al., 1996). Since only the ligand binding domain was expressed, bound to the cell membrane by a GPI anchor, binding occurred but signaling did not as judged by accumulation of Armadillo levels. In this respect Dfz2-GPI could act as a dominant negative for Wg signaling. In order to test this hypothesis *in vivo* transgenic flies were created carrying the Dfz2-GPI construct under the control of the Upstream Activation Sequence (UAS) promotor. By crossing these flies to various Gal4 stocks the effects of Dfz2-GPI overexpression could be examined in different tissues and different developmental stages where Wg activity is required.

wg mutant embryos are characterized by the expansion of the denticle belts on the ventral epidermis (Figure 1B) (reviewed by Perrimon, 1994). In fact, these embryos lose the regularly spaced belts and instead show a "lawn" of denticles that obscures both segmental and parasegmental boundaries. Overexpressing Dfz2-GPI with Da-Gal4 resulted in a phenotype similar to that seen in loss of function *wg* mutations (Figure 5B). In these embryos a phenotype similar to weak/moderate *wg* mutants was observed. Although the normal pattern of denticles can be seen there is clearly an expansion of the domain of denticles into the portion of the parasegment that is normally void of such structures. This phenotype suggests that the GPI anchored version of Dfz2 can non-productively bind Wg in the cells where its activity is required for the generation of

Figure 5. Wg Signaling is Inhibited by Overexpressing Dfz2-GPI.

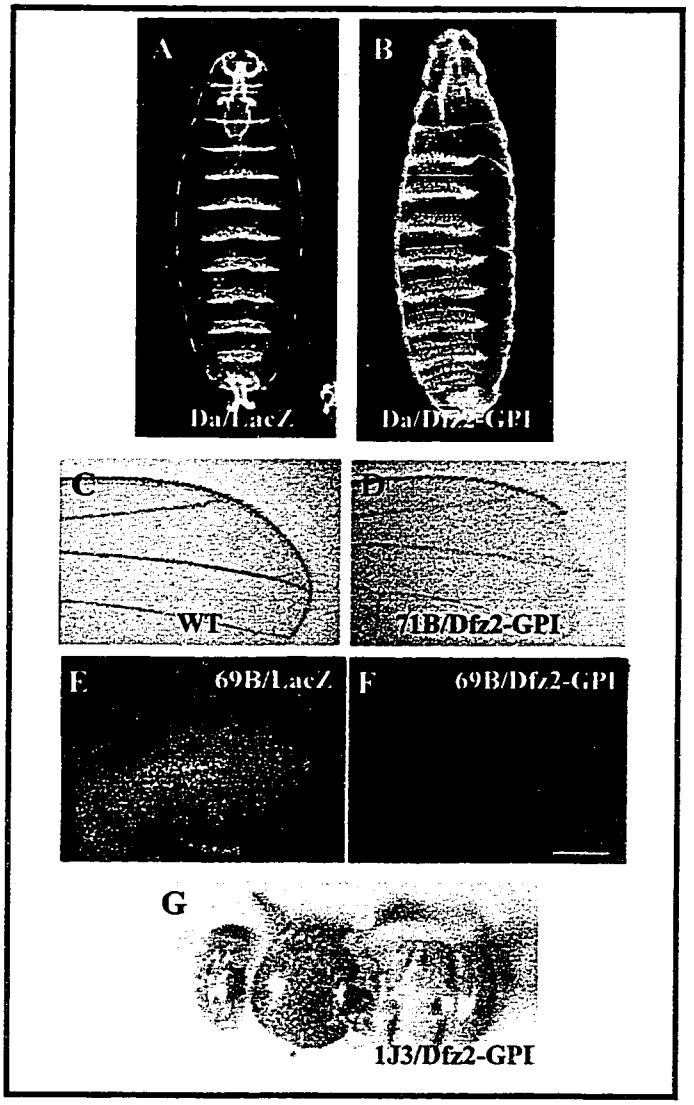

Figure 5. Wg Signaling is Inhibited by Overexpressing Dfz2-GPI.

Overexpression of Dfz2-GPI by several Gal4 drivers inhibits Wg signaling and results in phenotypes similar to *wg* loss of function mutants. Embryos reared at 29°C show an expansion of denticle belts in areas where Wg is necessary for the formation of naked cuticle (B) whereas overexpression of LacZ does not (A). Adult wings also show loss of *wg* phenotypes when Dfz2-GPI is expressed throughout the developing wing disc (D). A wild type wing is shown for comparison and is identical to wings where LacZ is overexpressed under the same conditions as Dfz2-GPI (C). The phenotype of the adult wing can be attributed to loss of activation of Wg target genes. Achaete (Ac) in green and Distal-less (Dll) in red (overlap is yellow) are expressed in the wing pouch of the wing imaginal disc in overlapping yet distinct patterns (E). However, when Dfz2-GPI is overexpressed throughout the wing pouch the expression of these genes is greatly reduced (F). In panels E and F, ventral is at the top and anterior is to the left. The Ac expression that remains in F is not Wg dependent. When Dfz2-GPI is overexpressed using the 1J3-Gal4 driver, the few surviving adults frequently lack wings (G).

naked cuticle. However, since the observed phenotype only resembles a weak/moderate wg phenotype, this suggests that the non-productive binding by the Dfz2-GPI is not complete and that some signaling occurs.

Because Dfz2-GPI acted as an inhibitor of Wg signaling in the embryo its effects in other tissues were examined. Under normal conditions Wg induces cell proliferation at the wing margin during larval and pupal development of the wing imaginal disc and specifies bristles at the wing margin. When Wg function is reduced or absent at this time, the resulting adult wing lacks a defined margin with its associated bristles or lacks wings altogether (Couso et al., 1994). When Dfz2-GPI was overexpressed throughout the wing disc using 71B-Gal4, it was observed in adults a phenotype similar to *wg* loss of function mutations (Figure 5D). The margins of these wings were scalloped in appearance indicating that growth at the margin was inhibited. The severity of this phenotype could be increased using different Gal4 drivers. For example, crossing Dfz2-GPI to 1J3-Gal4 frequently resulted in adults with only remnants of wings or no wings at all (Figure 5G).

As in the wing, Wg promotes outgrowth as well as dorsal/ventral patterning in the leg (Wilder and Perrimon, 1995). Overexpressing Dfz2-GPI in the leg with 1J3-Gal4 resulted in foreshortened legs usually involving loss of tibial structures or in some cases loss of the entire leg (data not shown). These data are consistent with those seen when *wg* is removed from this developing tissue.

To better understand the phenotype seen with Dfz2-GPI overexpression in adult wings the distribution of several positive markers of Wg signaling in the wing disc were

examined. Achaete (Ac) and Distal-less (Dll) protein are both expressed in the presumptive wing pouch of the wing imaginal disc (Neumann and Cohen, 1997). However, their expression patterns are different in several ways. First, Ac is activated by high concentrations of Wg whereas Dll does not require high levels in order to be activated (Neumann and Cohen, 1997). Second, the areas of expression of these Wg targets are spatially different in wing discs. Ac is expressed in a row 1-2 cells wide on either side of the Wg expressing cells in the anterior portion of the wing blade (Figure 5E). Dll on the other hand is expressed throughout the wing pouch with highest levels near the Wg source and decreasing further from the margin (Figure 5E). By overexpressing Dfz2-GPI throughout the wing disc, expression of both Ac and Dll are reduced significantly (Figure 5F). Note that the Ac expression that is not reduced in the dorsal and anterior portion of the disc is not Wg dependent (Phillips and Whittle, 1993).

The results of these experiments indicate that the CRD domain is sufficient to block Wg signaling in a variety of developing tissues. Since the transmembrane and cytoplasmic domains were not present in these experiments they most likely provide the signaling abilities of Dfz2. It is concluded that the CRD domain of Dfz2 can nonproductively bind the Wg ligand *in vivo*.

Full Length Dfz2 Expands Wg Targets in a Wg Dependent Manner

The results seen with Dfz2-GPI indicated that Wg signaling was inhibited. Therefore the effects of overexpression of the full length form of Dfz2 were examined. Because *wg* is essential for normal embryonic development, the first overexpression experiments targeted the embryo. The pattern of denticles in the anterior portion of each parasegment

is also altered when Wg is ectopically overexpressed (Noordermeer et al., 1992) (Figure 1C). In embryos where Wg is overexpressed these denticle belts fail to form and the ventral side of the embryo is said to have a "naked" phenotype. Overexpression of the full length form of Dfz2 by the embryonic Gal4 driver Da produced no effects on the ventral cuticle pattern of the embryonic epidermis (Figure 6G). The fact that a *wg*-like phenotype was not seen suggests that the Wg ligand is the limiting factor in patterning of the epidermis in this case.

Despite the negative result seen in the embryo, the effects of full length Dfz2 overexpression were examined in adult structures. Normally the adult wing blade is decorated with several types of mechanosensory bristles along the margin and the formation of these bristles is dependent on Wg function (Couso et al., 1994). These bristles can be found in ectopic areas of the wing blade when activators of the Wg pathway are ectopically produced in clones (Couso et al., 1994). When the full length form of Dfz2 was overexpressed throughout the wing blade an expansion of the domain that these bristles occupy was seen (Figure 6B). Interestingly, these bristles were only seen near the margin and not in the center of the wing. Since the formation of these bristles requires high levels of Wg, it is concluded that these levels of Wg are not attained further into the wing.

As in the wing, Wg is important in the leg for the growth and development of these appendages (Wilder and Perrimon, 1995, Penton and Hoffman, 1996). Overexpression of the full length protein resulted in duplicated leg structures most commonly seen in the tarsi (Figure 6H) but in some cases duplications including the whole leg were observed.

Figure 6. Overexpression of Full Length Dfz2 Activates Wg Targets.

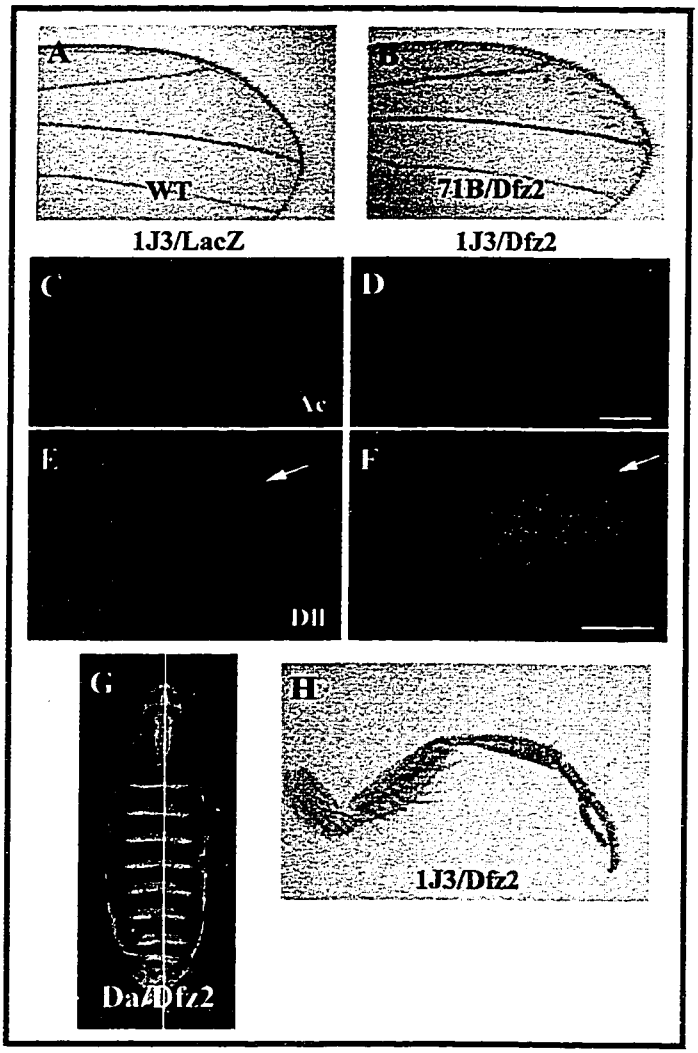

Figure 6. Overexpression of Full Length Dfz2 Activates Wg Targets.

Adult wings are decorated with mechanosensory bristles along the margin of the wing and their formation is Wg dependent. In wild type wings (A) these bristles are only found along the margin but when Dfz2 is overexpressed in wing discs these bristles are found at a distance from the wing margin (B) indicating that target genes are being activated. The wild type pattern of both Ac and Dll expression (C and E) is expanded in wing discs overexpressing Dfz2 (D and F). In panels C-F, ventral is at the top and anterior is to the left. Although loss of denticle belts was not seen in embryos overexpressing Dfz2 (G) as is seen when Wg is overexpressed, Wg overexpression like phenotypes in other tissues were observed. In the leg, overexpression of Dfz2 resulted in axis duplication of the leg (H), a phenotype observed when Wg is overexpressed in this tissue.

Overexpression of Dfz2 in the wing indicated that Wg targets were being activated. Therefore, molecular markers were used in the wing discs to examine the nature of the adult phenotype. When full length Dfz2 was overexpressed throughout the wing pouch the expression domains of both Ac and Dll were increased. (Figure 6D, F) Again, only the Wg dependent Ac expression was affected by increasing Dfz2 levels. In addition, other experiments revealed that the levels of these Wg responsive targets were not elevated when functional Wg was eliminated in the presence of excess Dfz2 (Cadigan et al., 1998). In these experiments the Wg^{ts} allele was used to reduce Wg levels in wing discs where Dfz2 was overexpressed. These discs looked similar to those where the GPI form of Dfz2 was overexpressed in that both Ac and Dll expression was severely reduced (see Figure 5F). Therefore it can be stated that the increase of Wg targets seen when full length Dfz2 was overexpressed, was Wg dependent and increased through additional Dfz2.

Dfz2 Stabilizes Wg

The results described above indicate that overexpressing Dfz2 was able to induce ectopic Wg signaling. In wing discs Wg acts a morphogen (Zecca et al., 1996; Neumann and Cohen, 1997). It is secreted from a row of cells at the presumptive margin of the wing disc and diffuses from this point to activate certain long and short range targets (Neumann and Cohen, 1997). In this model short range targets require high levels of Wg signaling while long range targets only require low levels of Wg signaling. It was observed that the expression of Dfz2 is inverse to that of Wg (Figure 7G and Cadigan, et

al., 1998). This pattern appears to be graded in fashion in that Dfz2 expression is lowest where Wg is highest. It was also observed that Wg regulates the expression of *Dfz2* (Cadigan, et al., 1998). In wing discs expressing a temperature sensitive form of Wg, that renders the protein inactive at 29°C , *Dfz2* expression expanded when larvae were reared at this temperature 24 hours prior to fixation (Cadigan et al., 1998). In addition, activated forms of Arm that phenocopy Wg overexpression, reduced endogenous *Dfz2* expression when overexpressed in wing discs (Cadigan, et. al., 1998). Thus, Wg regulation of *Dfz2* may help maintain the differential activation of target genes. In order to study this possibility more closely the distribution of Wg was examined when Dfz2 was overexpressed in the wing disc.

Normally Wg diffuses from a row of 3-4 cells, expressing *wg* mRNA, along the wing margin out to a distance of 25 cells (Neumann and Cohen, 1997; Cadigan et al., 1998). As the protein diffuses it is clear that a gradient is established with highest levels close to the source (Figure 7A). However, overexpression of both Dfz2 and Dfz2-GPI disrupt this gradient and result in high levels of Wg further from its source than normal (Figure 7B, C). When Dfz2-GPI was overexpressed by two Gal4 drivers, Patched (Ptc) and 1J3, the range of Wg diffusion was dramatically increased (Figure 7C, D). However, the cells producing *wg* mRNA were not significantly increased (compare Figure 7A to B and C) indicating that the increased Wg levels are not due to increased Wg production. It is surmised that this pattern of accumulated Wg far from its source is due to the nonproductive binding nature of the GPI version of Dfz2. In contrast, full length Dfz2 (Figure 7B) results in Wg accumulation levels that are not as broad as those seen with

Dfz2-GPI, but are still greater than normal levels. In this case the full length Dfz2 protein may allow internalization and degradation of the ligand via vesicles and hence the range of accumulation is decreased.

Wg expression in wing discs is dynamic throughout the larval stages. Wg is expressed throughout the wing disc in early third instar larvae and ultimately resolves to the narrow stripe during late third instar (Cadigan et al., 1998). While the results described above indicate that Dfz2 stabilizes Wg, it was unclear whether the stabilized protein was from early third instar expression or late third instar expression. In order to address this question Western blots on wing discs expressing both forms of Dfz2 were performed. Using the temperature sensitive nature of Gal4, larvae were grown at 18°C in order to reduce transgene expression and then shifted to 29°C 24 hours prior to disc dissection of wandering third instar larvae. Two different Gal4 drivers, Patched (Ptc)- and 1J3-Gal4, were used. Discs were then used to determine the levels of Wg protein by Western blot analysis. Both Dfz2 and Dfz2-GPI showed higher levels of Wg protein as compared to controls (Figure 8). Under the control of each Gal4 driver the GPI form of Dfz2 was able to retain higher levels of Wg than the full length form. Although this result shows that Wg levels are higher when Dfz2 or Dfz2-GPI are overexpressed in wing discs, it does not completely rule out the possibility that low levels of Dfz2 expression at 18°C account for the increased accumulation of Wg. However, experiments where clones of cells overexpressing Dfz2 were made in late third instar larvae Wg accumulated to high levels

Figure 7. Both Dfz2 and Dfz2-GPI Stabilize Wg.

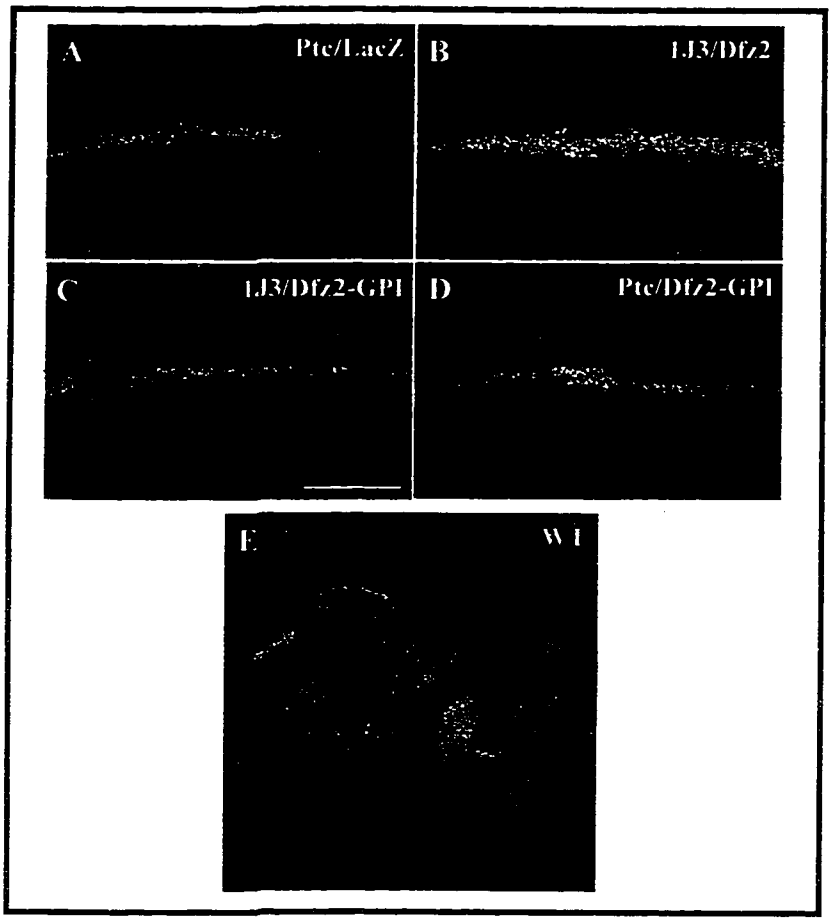

Figure 7. Both Dfz2 and Dfz2-GPI Stabilize Wg.

In all panels (except G) *wg* RNA is in red and Wg protein in green (overlap is yellow). Overexpressing Dfz2 and Dfz2-GPI resulted in Wg accumulating to high levels far from its source. Normally Wg levels are highest in an area 2-3 cells from its site of synthesis and then rapidly drops off but is still present up to 25 cell diameters away (A). Dfz2 (B) and Dfz2-GPI (C), when overexpressed, can stabilize Wg as it moves from its source in the wing disc by binding to Wg and preventing its degradation. Because full length Dfz2 can signal, Wg is most likely internalized and thus does not accumulate to the levels seen with Dfz2-GPI which binds Wg in a nonproductive manner. Patched (Ptc)-Gal4 is expressed along the anterior/posterior axis of the wing disc and accumulates Wg to dramatic levels when Dfz2-GPI is overexpressed (D). Wg (green) and Dfz2 (red) expression patterns are inverse to each other in normal wing, leg, and halter discs (G).

Figure 8. Western Blot Analysis of Increased Wg in Discs Overexpressing Dfz2 and Dfz2-GPI.

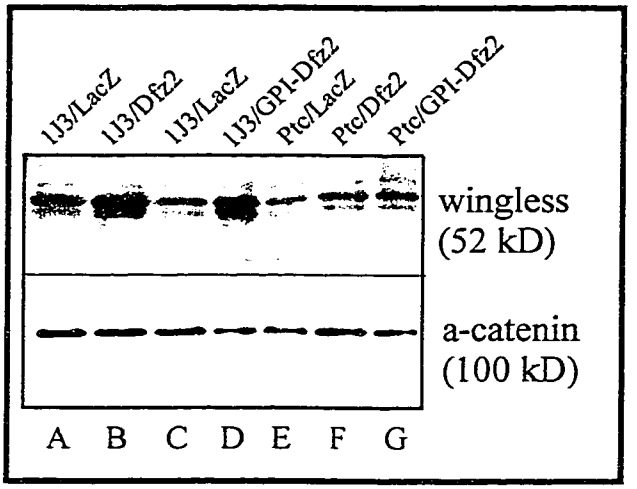

Figure 8. Western Blot Analysis of Increased Wg in Discs Overexpressing Dfz2 and Dfz2-GPI.

Both Dfz2 and Dfz2-GPI overexpression result in increased levels of Wg in wing discs as compared to controls. Two different Gal4 drivers were used to overexpress both forms of Dfz2. In each case the GPI form of Dfz2 resulted in higher Wg levels than the full length form (compare lanes B and D, and lanes F and G). In addition, Wg levels seen with the full length form of Dfz2 are higher than controls (compare lanes A and B, and lanes E and F). Alpha-catenin is shown as a loading control. The signals were quantitated by densitometry, (Cadigan et al., 1998) and the ratio of Wg/alpha-catenin signal (in arbitrary units with the control lanes A, C, and E normalized to 1.0) were as follows: lane B, 1.7; lane D, 5.6; lane F, 1.3; and lane G, 2.2. The control lanes C and E are 52% and 44% of that in lane A.

only in the Dfz2 expressing cells (Cadigan et al., 1998). Taken together, these results indicate that Dfz2 stabilizes Wg as it is secreted from its source during late third instar.

X-ray Mutagenesis of P0469 Removes Dfz2

The experiments described above have shown that overexpressing Dfz2 can influence Wg signaling *in vivo*. However, these results do not show that Dfz2 is essential for Wg signaling and the possibility remains that Dfz2 works in conjunction with other factors to transduce the signal. Therefore, analysis of *Dfz2* loss of function mutants would further establish the role of this protein in Wg signaling. However, there are currently no *Dfz2* mutants to examine. To resolve this issue we mutagenized a w^+ P-element close to *Dfz2* in order to create small deficiencies at the *Dfz2* locus (Figure 9A). X-ray mutagenesis typically results in small deletions and chromosome rearrangements (Grigliatti, 1986) so this method was used to delete the P-element along with *Dfz2* (Figure 9B)/ A total of 20 lines showing a loss of w^+ (P0469) were established from screening approximately 26,000 F1 progeny. Thirteen of these lines were found to have lethal excisions and were tested for complementation with other nearby lethal mutations. Lethal excisions were used for complementation analysis based on the hypothesis that loss of the Wg receptor would be lethal just as loss of *wg* itself is lethal.

Complementation analysis (Table 1) indicated that 10 of the 13 lethal lines had small lesions near and perhaps including *Dfz2*. Each of these lines failed to complement only one (and in one case two) of the local lethals, or failed to complement line 0469-2 which was shown to be deficient of *Dfz2* by *in situ* hybridization to embryos. From these data a

map of the region was constructed including the local lethals and some of the excision lines (Figure 10).

Analysis of polytene chromosomes was used to determine if *Dfz2* had been deleted in these 10 lethal lines. Orcein staining was used to characterize the nature of the lesions created by X-ray mutagenesis. Three lines (0469-2, 0469-19, and 0469-20) were shown to delete the band 76A (Figure 11A), one line (0469-6) deleted bands 75F to 76A (not shown), and one line (0469-16) appeared to be an inversion spanning bands 71F to 76A (not shown). The remaining five lines showed compound rearrangements. To confirm that *Dfz2* had been deleted *in situs* were performed on polytene chromosomes. Lines 0469-2 (shown), 0469-6, 0469-19, and 0469-20 were found to be deficient of *Dfz2* (Figure 11B), and line 0469-16 was found to have a potential inversion in *Dfz2* (data not shown).

Since 0469-2 appeared to be a small deficiency that removed *Dfz2*, we used this line to test for Dfz2 protein staining in embryos. The parent stock for the X-ray mutagenesis, P0469, shows Dfz2 staining in the axon tracts of the central nervous system and the anterior and posterior constrictions of the midgut (Figure 11C). Because line 0469-2 is lethal and requires a balancer chromosome to maintain the viability of the mutant chromosome, only one-quarter of the embryos examined should be homozygous for the 0469-2 chromosome. As predicted, approximately one-quarter of the embryos of line 0469-2 show no Dfz2 staining (Figure 11D). The remaining embryos show the same Dfz2 staining as the parent stock.

Figure 9. X-Ray Mutagenesis Scheme.

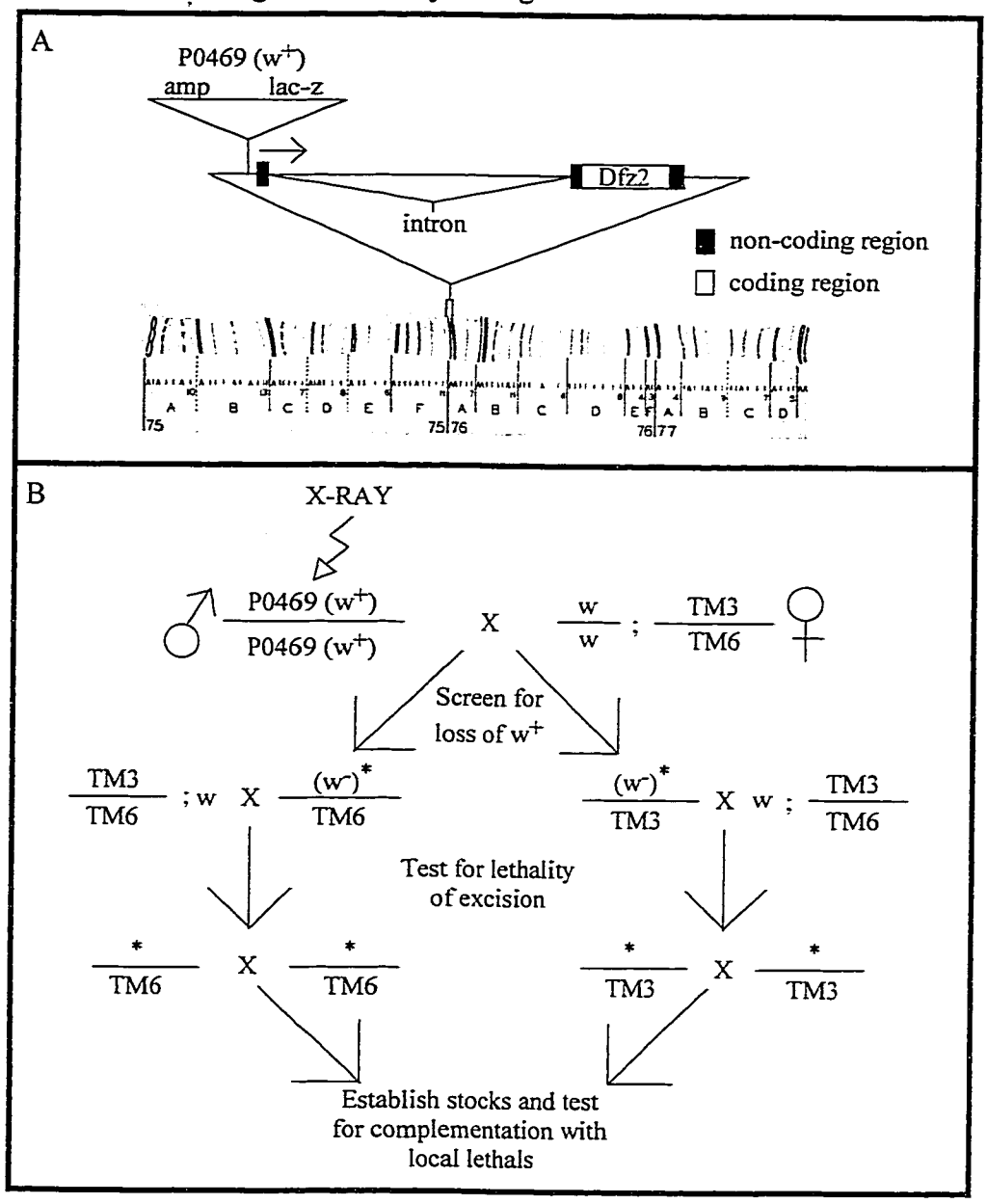

Figure 9. X-Ray Mutagenesis Scheme.

The stock P0469 contains a P-element located 60 bp from the transcriptional start site of Dfz2 determined by sequence data from excision of the P-element. This stock was used as a start point for an X-ray mutagenesis screen. The cytological location of *Dfz2* was placed at 76A1-4 by *in situ* hybridization to polytene chromosomes (A). Third chromosome w^- balancer stocks were used to detect and maintain lethal excisions of the P-element. The screen was based on the loss of pigmented eyes provided by w^+ from the P-element of the mutagenized P0469 males (B).

Table 1. Complementation Data.

Excision	Viability	Local Lethals nkd	P1706	5A8	neo 27	Df VW3
0469-1	L	C	C	C	C	C
0469-2	L	C	NC	C	C	C
0469-3	V					
0469-4	L	C	C	C	C	C
0469-5	SL	C	C	C	C	C
0469-6	L	NC	NC	C	C	C
0469-7	L	C	C	C	C	C
0469-8	V					
0469-9	V					
0469-10	L	C	C	C	C	C
0469-11	L	C	C	C	C	C
0469-12	V					
0469-13	L	NC	C	C	C	C
0469-14	L	C	NC	C	C	C
0469-15	V					
0469-16	L	C	C	NC	C	C
0469-17	SL	C	NC	C	C	C
0469-18	L	C	C			C
0469-19	L	C	NC	C	C	C
0469-20	L	C	NC	C	C	C

Key:
L-Homozygous lethal
V-Homozygous viable
SL-Semi-lethal
C-Complementation
NC-Non-complementation
SC-Semi-complementation

	Excisions 1	2	4	5	6	7	10	11	13	14	16	17	18	19
0469-1	X													
0469-2	C	X												
0469-3														
0469-4	C	C	X											
0469-5	C	SC	C	X										
0469-6	C	NC	C	C	X									
0469-7	C	C	C	C	C	X								
0469-8														
0469-9														
0469-10	C	C	C	C	C	C	X							
0469-11	C	NC	C	NC	NC	C	C	X						
0469-12														
0469-13	C	NC	C	C	NC	C	C	NC	X					
0469-14	C	NC	C	C	NC	C		C	NC	X				
0469-15														
0469-16		C	C	C	C	C	C	C		C	X			
0469-17	C								C	NC		X		
0469-18														
0469-19	C								C	NC		NC		X
0469-20	C								C	NC		NC		NC

Table 1. Complementation Data.

Of the 20 excision lines obtained by X-ray mutagenesis, 13 were found to be lethal. These lines, and two lines showing partial lethality, were crossed to local lethal stocks and to each other to determine the size and position of the X-ray lesion. Most of the lethal lines failed to complement P1706. Lines 0469-2, 0469-19, and 0469-20 appear to be small deletions because they only fail to complement one of the local lethals.

Figure 10. Proposed Map of the Excision Lines and Local Lethals.

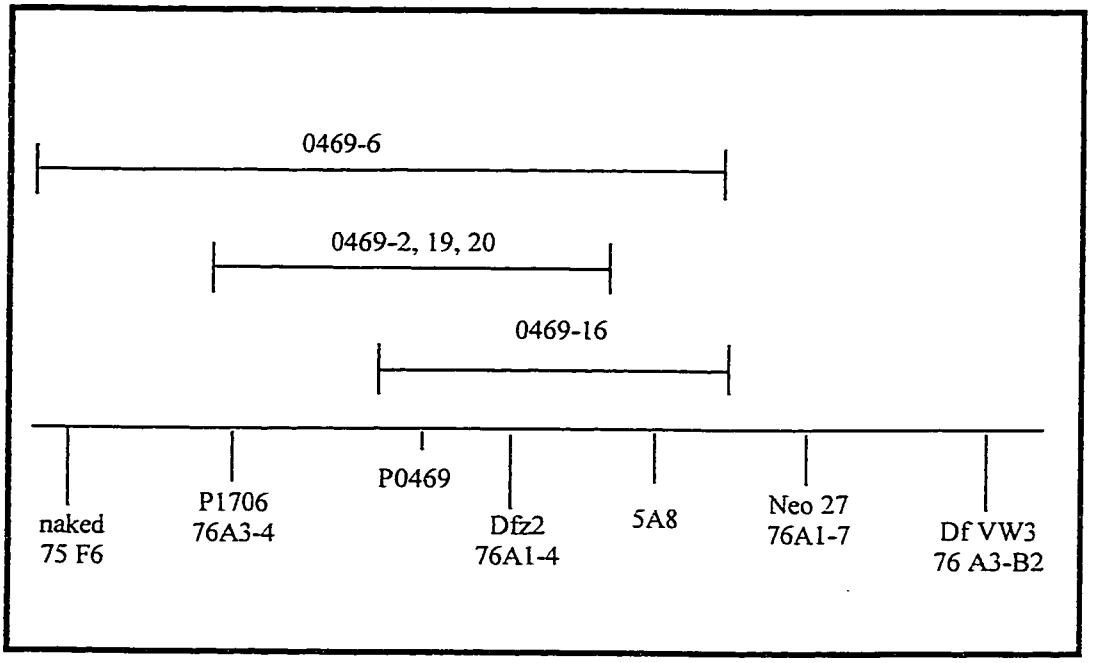

Figure 10 Proposed Map of the Excision Lines and Local Lethals.

Based on the complementation data a map of the local lethals and the mutagenized lines is suggested. Lines 0469-2, 0469-19, and 0469-20 appear to be the smallest deletions because they fail to complement only P1706. Each of these lines was shown to lack *Dfz2* by polytene chromosome *in-situ*. Line 0469-6 also lacks *Dfz2* but appears to be a larger deletion because it fails to complement *naked* at 75F6. Although line 0469-16 appears to be a small deletion by complementation analysis, polytene chromosome analysis shows that the lesion is an inversion with one break point in *Dfz2*.

Figure 11. Polytene Chromosome Analysis and Embryo Antibody Staining of 0469-2.

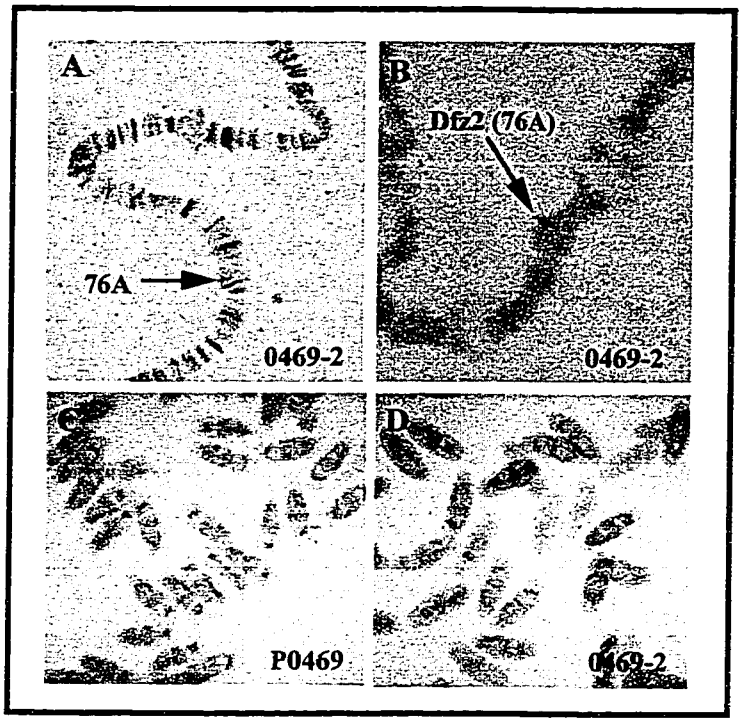

Figure 11. Polytene Chromosome Analysis and Embryo Antibody Staining of 0469-2.

Attention was focused to line 0469-2 because it appeared to be a small deletion based on complementation data. Orcein staining of this line showed that X-rays had induced a deletion of band 76A (A). Probing with Dfz2 cDNA *in situ* hybridization confirmed that 0469-2 lacked *Dfz2* (B). Loss of Dfz2 protein was confirmed by antibody staining of embryos with an antibody that recognizes the C-terminus of the protein. The parent P-element stock P0469 shows normal Dfz2 staining in all embryos (C), whereas 0469-2 shows lack of staining in approximately ¼ of the embryos (D).

Although several lines obtained from this X-ray mutagenesis clearly remove *Dfz2*, complementation analysis indicates that these deletions take out additional genes as well. However, these lines are clearly useful for several reasons. First, the small deletions of lines 0469-2, 0469-19, and 0469-20 will be used as a start point for an EMS screen directed at *Dfz2*. Lethal mutations obtained from an EMS mutagenesis crossed to any of these lines may be specific mutations in *Dfz2*. Second, these lines can be analyzed for phenotypes in the embryo. If embryos of these lines show phenotypes similar to those of *wg* mutant embryos then, perhaps, a specific mutation in *Dfz2* has been obtained. Third, these lines can be tested in backgrounds lacking *fz*, another member of the Frizzled family in *Drosophila*. Analysis of embryos mutant for both Frizzleds may help clarify the requirement for one or both of the genes during early development.

Discussion

Wingless Signaling in *Drosophila*

Wnt signaling plays an important role in the development of several different animal systems. In the mouse Wnt signaling is necessary for proper CNS formation (Thomas et al., 1991), and in *Xenopus* Wnt signaling affects body axis formation (McMahon and Moon, 1989). In *Drosophila*, the *Wnt* gene *wingless* (*wg*) is necessary during several distinct developmental periods. In the embryo, Wg is necessary to ensure that proper parasegmental boundaries are established and maintained and that the cuticular denticles are inhibited in the posterior of each segment (Noordermeer et al., 1992). In the imaginal discs, Wg is necessary for growth and cell fate specification during larval and pupal

development (Wilder and Perrimon; 1995, Neumann and Cohen, 1997). Activation of the *wg* pathway during these developmental periods results in embryos showing a loss of denticles and ectopic adult structures while inactivation of the pathway via *wg* mutants results in embryos with expanded denticles and adults lacking properly developed appendages.

The Wg protein is a secreted molecule and acts as a morphogen (Zecca et al., 1996) by regulating gene expression differentially as it is secreted from its source. However, a candidate receptor through which this ligand transduces a signal has only recently been identified (Bhanot et al., 1996). Loss of function *fz* mutations show a polarity defect in the hairs of the wings and notum of adult flies (Krasnow and Adler, 1994). And specific mutations in *dishevelled* (*dsh*) also produce polarity defects in these tissues (Krasnow et al., 1995). Additionally, Wg is known to require Dsh in order to signal (Klingensmith et al., 1993). This implied that Wg and Fz share a common component that is involved in cell-cell signaling. However, *fz* mutants do not show *wg* mutant like phenotypes. Therefore, a search for *fz* related genes resulted in the identification of *Dfz2* (Bhanot et al., 1996). Dfz2 was shown to bind and transduce the Wg signal in cell culture experiments (Bhanot et al., 1996). Although similar proteins such as mouse Frizzled 8 (Mfz8) could bind and Frizzled (Fz) could transduce the Wg signal in these experiments, they were unlikely candidates due to their loss of function phenotypes. The experiments described above were conducted to determine whether or not Dfz2 could function as the Wg receptor *in vivo*.

The Role of the CRD Domain of Dfz2

A common element of the Frizzled family of receptors is the invariant location of 10 cysteine residues in the extra cellular domain (Xu and Nusse, 1998) referred to as the cysteine rich domain (CRD). It has been shown that the CRD is capable of binding Wg and that it can prevent signaling from occurring by binding the ligand nonproductively. However, the CRD of Fz can also bind Wg and has been shown to act as a dominant negative for Wg signaling in the wing (Zhang and Carthew, 1998). In these experiments overexpressing the CRD of Fz resulted in scalloped wings similar to those seen when the CRD of Dfz2 was overexpressed. Interestingly, coexpression with full length Fz did not rescue the phenotype, but coexpression with full length Dfz2 did. This suggests that while the CRD of Fz can bind Wg and inhibit signaling, Dfz2 is necessary to transduce the signal. Therefore the question arises as to whether Dfz2 and Fz compete for the Wg ligand *in vivo*. If this is the case then one would expect to see Wg like phenotypes when full length Fz is overexpressed. This hypothesis has been tested and only planar polarity phenotypes are observed when Fz is overexpressed in the wing (Krasnow and Adler, 1994; M.F. unpublished observations). In this respect, the transmembrane and cytoplasmic domains of Dfz2 may be the important component of Wg signaling. These domains may confer the properties that ensure only Wg is bound and by deleting these domains this stringency is lost.

Full Length Dfz2 Can Function as a Wg Receptor

It has been shown that Dfz2 can act as a Wg receptor *in vivo*. The expression of Wg responsive target genes such as Achaete (Ac) and Distal-less (Dll) was increased

ectopically when the full length form of Dfz2 was expressed throughout the imaginal wing disc. The result of this overexpression could also be seen in adult structures such as wings and legs where ectopic bristles and duplications, respectively, were observed. Each of these results resembles those seen when Wg is ectopically overexpressed. When Wg was reduced, the increase in target gene expression was lost indicating that Wg acts through Dfz2 and that Dfz2 requires Wg for its activating properties (Cadigan et al., 1998).

Although Fz has been shown to act as a Wg receptor in cell culture (Bhanot et al., 1996) the study was unable to produce Wg overexpression effects in the wing or leg by overexpressing Fz. In the wing only a planar polarity effect (orientation of wing hairs) was seen and no effects were seen in the leg. This suggests that while Fz can function as a Wg receptor in cell culture, it does not have this ability in wing or leg discs. The difference in the ability of Fz and Dfz2 to transduce the Wg signal may be due to "specificity factors". These factors may permit only polarity signals to be transduced through Fz and only differentiation signals to be transduced through Dfz2 as suggested by Zhang and Carthew (1998). In addition, these factors may be regulated differently in different tissues such that Fz can transduce the differentiation signal in some tissues but not all tissues.

Oddly, the study did not observe an effect when Dfz2 was overexpressed in the embryo using the strongest transgenic lines and Gal4 drivers at our disposal. Dfz2 expression in the embryo is graded in fashion and lowest close to where Wg is expressed (Muller et al., 1999). Although a similar pattern in was observed in wing discs (low

Dfz2 expression where Wg is high) it is possible that the amount of Wg secreted during embryonic development is not sufficient to activate targets at increased distances even when its receptor is overexpressed.

Morphogen Stabilization by Dfz2

Morphogens are considered to be molecules that emanate from a source , diffuse across a range of cells, and can influence cell fate. Within this range of cells, the morphogen can affect cells in a concentration dependent manner. Wg acts as a morphogen in the wing disc by activating short range targets such as Ac at high levels of Wg and long range targets such as Dll at low levels of Wg (Neumann and Cohen, 1997). The study has shown that Dfz2 helps to stabilize Wg in the wing disc. By overexpressing Dfz2, Wg can accumulate to high levels further from its source than is normal. It is believed that this excess Wg is from late third instar expression based on Western blot examination (Figure 8) and clonal analysis (Cadigan et al. 1998) of Dfz2 in wing discs. Wg expression levels are inverse to that of Dfz2 expression levels (Figure 7G), this relationship may prevent Wg accumulation close to its source and limit the extent of short range target expression. As Wg diffuses from its source, nearby cells have low levels of receptors which allow its diffusion and degradation as the protein moves through the field of cells. As fewer Wg molecules travel farther within this field they are more effectively stabilized by increasing receptor levels. In this manner, high levels of *Dfz2* far from the source of Wg may facilitate long range target induction.

Dfz2 Mutants

The deficiency line 0469-2 removes *Dfz2* and this is perhaps the first small deficiency to remove this gene. Polytene chromosome analysis and embryo staining confirm that both the *Dfz2* locus at 76A1-4 and Dfz2 protein, respectively, are absent in this line. Although it appears that other genes are removed as well this line is important for future studies on the role of Dfz2 in Wg signaling. One can use 0469-2 as a start point for future EMS mutagenesis screens to create point mutations in *Dfz2*. Creating a point mutation in Dfz2 that results in a non-functional protein will help to clarify the role of Dfz2 in the Wg pathway.

Although embryos mutant for Dfz2 that have been created do not show a cuticle phenotype (Matt Fish and Ken Cadigan, unpublished observations), it is possible that other members of the Frizzled family can replace the function of Dfz2 in the embryo. In fact, double mutants of fz and Dfz2 have been made and embryos of this genotype show cuticle phenotypes similar to Wg mutant embryos (Muller et al., 1999). This suggests that both Dfz2 and Fz function in the Wg pathway in the embryo.

Redundant Function of Dfz2 and Frizzled

The question of redundant receptors arises when one examines the effects of mutations in *Dfz2* and *fz*. Neither *Dfz2* nor *fz* mutants show a cuticle phenotype alone. However, when both maternal and zygotic *fz* and zygotic *Dfz2* are removed from the embryo a strong *wg* like phenotype is observed (Muller et al., 1999). In addition, Kennerdell and Carthew (1998) have shown that injecting embryos with double stranded *fz* and *Dfz2* RNA results in *wg*-like phenotypes. No phenotype is seen when either double stranded

RNA is injected alone. In the embryo then, it appears that Dfz2 and Fz have a redundant function.

In contrast to the embryo, the wing disc does not appear to require Fz function for Wg signaling. When Dfz2-GPI is overexpressed in the wing disc adult wings are scalloped along the wing margin. When this same experiment is done in a *fz* mutant background there is no enhancement of the phenotype (Ken Cadigan, unpublished data). Therefore, it is suggested that another factor is involved in the interaction between Wg and its receptor.

Wingless signaling in *Drosophila* and Wnt signaling in other animals has profound consequences when perturbed. While it appears that members of the Frizzled family of receptors can transduce Wnt signals, their exact relationship is not known. In *Drosophila* there are four different *Wnt* genes and at least three genes in the *frizzled* family. In the mouse the numbers are even greater. Determining how the proteins interact with each other in different developmental context will be a challenge.

Experimental Procedures

Dfz2 and Dfz2-GPI Subcloning

The full length Dfz2 cDNA (Gene Bank Accession Number U65589) from M. Brink (Bhanot et al., 1996) was subcloned from pMK 33 (Koelle et al., 1991) into the pUAST vector (from Dererk Lessing) (Brand and Perrimon, 1993). The Dfz2-GPI cDNA from C. Harryman-Samos (Bhanot et al., 1996) was subcloned from pRK 5 (Schall et al., 1990) into the pUAST vector. Briefly, the *Spe*I and *Xho*I restriction sites were used to cut the full length 2.2 Kb Dfz2 cDNA from pMK 33 and this insert was ligated into the *Xba*I and *Spe*I sites of pUAST. The *Cla*I and *Apa*I restriction sites were used to cut the 1.0 Kb Dfz2-GPI cDNA from pRK 5 and the ends were filled with T4 Polymerase. This insert was then ligated into pUAST that was also blunt ended and dephosphorylated at the *Xho*I site. *E. coli* was transformed by heat shock at 37°C for three minutes with the ligated plasmids and colonies containing the insert in the proper orientation were selected by restriction digest analysis of the newly created pUAST P-elements. *Xho*I and *Sph*I restriction sites were used to detect a 2 Kb fragment indicating correct orientation of the full length insert. *Xho*I and *Not*I restrictions sites were used to detect a 1 Kb fragment indicating correct orientation of the GPI insert. One positive colony was selected for each P-element and was grown in LB broth to ensure at least 24 *u*g of each plasmid could be purified. Plasmids were purified using the Invitrogen S.N.A.P. miniprep kit and each plasmid was diluted to a concentration of 1*ug/ul* of water (Invitrogen, San Diego, CA).

Transgenic Flies

Transgenic flies were made using the method of Rubin and Spradling (1982). Briefly, the plasmids were mixed with Delta 2-3 plasmid from A. McCormick which contains a source of transposase activity (Laski et al., 1986). This injection cocktail consisted of either full length Dfz2 or Dfz2-GPI plasmids at a concentration of 600 *ng/u*l and the Delta 2-3 plasmid at a concentration of 150 *ng/u*l in water. Approximately 1-2% of egg volume of each injection cocktail was injected into approximately 300 preblastoderm stage embryos of w^{1118} *Drosophila melanogaster* hosts and the surviving embryos were reared to the adult stage. These adults were then back-crossed to the stock w^{1118} and the progeny screened for the presence of w^{+} (from the pUAST vector) red eyes indicating successful P-element insertion. Male flies from individual crosses giving progeny showing w^{+} eyes were crossed to flies of *w* balancer lines to determine on which chromosome the P-element was inserted and to determine if the insertion was homozygous viable or not. For each construct at least 10 individual lines were established and approximately half of these were homozygous viable. All of the UAS lines derived from injection of each plasmid were compared to each other for strength of expression of the UAS construct as determined by their phenotypes. Experiments described were performed with strongly expressing constructs unless otherwise noted.

Overexpression of Dfz2 or Dfz2-GPI with Gal4 Enhancer Trap Lines

Overexpression of the transgenes was accomplished using the method of Brand and Perrimon (1993). Briefly, the Gal4 gene has been inserted into genomic DNA, by P-element mediated transformation (Rubin and Spradling, 1982), under the control of

various genomic enhancers. Thus, the Gal4 gene, which is a yeast transcription activator, is expressed in tissue- and stage-specific domains of various Gal4 stocks. The pUAST vector contains five optimized Gal4 binding domains which facilitate transcription of genes downstream of the binding domains in the presence of Gal4. Transgenes can be inserted into the pUAST vector and then introduced into *Drosophila* hosts by P-element mediated transformation. By crossing fly lines carrying the pUAST-transgene to various Gal4 enhancer lines, transgenes can be expressed in the specific pattern of the Gal4 enhancer. The Gal4 enhancer trap "drivers" used were 69B- and 1J3-Gal4 (Brand and Perrimon, 1993), and 71B-, Ptc-, Da-, and En-Gal4 (Johnson et al., 1995). The Gal-4 drivers 69B- and 71B-Gal4 are expressed in the presumptive wing blade area of the wing imaginal disc and 1J3-Gal4 is expressed throughout the wing disc and the leg discs as well as in the embryo. In the wing disc, Ptc-Gal4 is expressed in a stripe of cells along the anterior-posterior boundary. Engrailed (En) -Gal4 and Daughterless (Da)-Gal4 are expressed throughout the embryo during early embryogenesis and continues until hatching.

Cuticle Preparations

Embryos from 24 hour collections were dechorionated in 60% bleach for 2 minutes and fixed in 4% formaldehyde in PBS (Sambrook et al., 1989) for 20 minutes. Embryos were then devitilinized in 1:1 heptane: methanol in an Eppendorf tube and transferred onto a microscope slide in 1:1 Hoyers:Lactic Acid media (Ashburner, 1989) at 60°C overnight under a coverslip to dissolve non-cuticle tissue. Cuticles were examined and photographed on a Nikon microscope.

Imaginal Disc Whole-Mount Immunostaining

Imaginal discs were dissected from wandering third instar larvae in PBS and fixed in 4% formaldehyde in PBS for 15 minutes. After washing in PBS/0.5% Triton X-100 (PBST) discs were incubated with primary antibodies in PBST/5% normal donkey serum at 4°C overnight . Affinity-purified rabbit anti-Wg antisera (1:50) was provided by C. Harryman-Samos (Nusse lab, Stanford Univ.). Purified rabbit anti-Distal-less (Dll) antisera (1:150) was from G. Panganiban (Carroll lab, Univ. Wisconsin-Madison). Mouse monoclonal anti-Achaete (Ac) hybridoma supernatant (1:4) was from K. Vorwerk and S. Carroll (Univ. Wisconsin-Madison). Mouse monoclonal anti-Dfz2 hybridoma supernatant (1:10) was from S. Pronovost (Nusse lab, Stanford Univ.). FITC, Cy3, and Cy5 conjugated secondary antibodies (Jackson Immuno-chemicals West Grove, PA) were diluted to a concentration of 1.5 mg/ml in the manufacturer's recommended buffer and then further diluted 1:200 in PBST/5% normal donkey serum and incubated on discs for 2 hours at room temperature. Discs were then washed (3 X 10 minutes) in PBST and mounted in Vectashield (Vector Labs, Burlingame, CA) on microscope slides. All fluorescent pictures were obtained with a Bio-rad MRC-1000 confocal laser coupled to a Ziess Axioscope and processed as Adobe Photoshop files.

Western Blot Analysis of Imaginal Discs

Discs were dissected from third instar larvae in cold PBS and transferred to Eppendorf tubes, where they were homogenized in SDS loading buffer with tight fitting pestles, heated to 100°C for 4 min, and then stored at –20°C until use. Immunoblotting was done as described by Willert et al. (1997) using mouse monoclonal anti-Wg supernatant

(1:100) provided by S. Cohen (European Molecular Biology Laboratory, Heidelberg, Germany) or rat monoclonal anti-alpha-catenin (1:1000; Oda et al., 1993) as loading control. The secondary antibodies were conjugated to horseradish peroxidase and detected with SuperSignal ULTRA Chemiluminiescent Substrate (Pierce, Rockford, IL) using the manufacturer's protocol.

Mutagenesis Screen

A stock containing a w^+ P-element 60 bp from the transcriptional start site of *Dfz2* (Ken Cadigan, personal communication) (Figure 9A) was excised by X-ray mutagenesis. This method was chosen because it typically creates small deletions or rearrangements in chromosomes (Grigliatti, 1986) Approximately 400 males (0 to 2 days old) of the stock P0469 (from the Nathans lab, John Hopkins Univ.)were given a 4000 rad dose in an X-ray cabinet machine. Mutagenized males were allowed to recover for several hours and crossed to a third chromosome *w* balancer stock. Progeny were screened for loss of w^+ indicating loss of the P-element and perhaps loss of the *Dfz2* gene (Figure 9B).

Complementation Analysis

Excision lines were tested for complementation against several stocks with other lethal mutations near *Dfz2*. The local lethal stocks tested were as follows: *nkd*, a lethal mutation in the *naked cuticle* gene at 75F6, P1706, a lethal P-element insertion at 76A3-4, 5A8, an uncharacterized mutation close to *Dfz2*, neo 27, a P-element insertion at 76A1-7, and VW3, a gamma ray induced deficiency encompassing 76A3-B2. *Dfz2* maps to the cytological location 76A1-4 (Figure 10). All stocks were obtained from the

Bloomington Stock Center, Bloomington IL, except 5A8 which was from K. Cadigan (Nusse lab, Stanford Univ.).

Polytene Chromosome Analysis

In situ hybridization and orcein stains were done on polytene chromosomes of 10 lethal excision lines to determine the effects of X-ray mutagenesis on the Dfz2 locus. A cDNA (from M. Brink, see "Dfz2 and Dfz2-GPI Subcloning" above) of *Dfz2* containing some 5' and 3' UTR sequence was used as probe for *in-situ* hybridization. Briefly, salivary glands were dissected from wandering third instar larvae in 0.7% NaCl and then fixed in 45% glacial acetic acid for 1 minute. Glands were then placed in a droplet of 3:2:1 glacial acetic acid:water:lactic acid on a coverslip for 5 minutes and then squashed to spread the chromosomes. Chromosomes were denatured in a 2X SSC (Sambrook et al., 1989) solution for 30 minutes, then 0.07M NaOH for 2 minutes. The squashes were incubated (in 4X SSC, Denhart's solution (Sambrook et al., 1989), 10% dextran sulphate, 0.02% denatured salmon sperm)with biotin labeled DNA probe overnight at 58°C. The probe was made by randomly labeling 100 ng of DNA (Sambrook et al., 1989) and approximately 50 ng of labeled probe was used for each squash. The probe was detected on the chromosomes by incubating the squashes with Avidin-horseradish peroxidase secondary antibody (Sigma, St. Louis, MO) (2.2 mg/ml) diluted 1:50 (in 0.1g BSA, 100ul 0.5M EDTA, 10 ml PBS) at 37°C for 30 minutes and developing with 0.5 mg/ml DAB/H_2O_2 (0.01% final concentration) at 37°C for 30 minutes.

Orcein stains were performed to determine the size and nature of the X-ray induced lesions. Salivary glands were dissected as described above and then squashed and spread in 45% glacial acetic acid/2% Orcein stain.

In all cases adults containing the mutagenized chromosome over the TM6 (Tubby) balancer were out-crossed to wild type flies to ensure that mutagenized chromosomes over wild type chromosomes could be selected for polytene chromosome analysis. Squashes were examined and photographed under a 100X objective using a Nikon microscope.

Embryo Antibody Staining

Overnight embryo collections were dechorionated in 60% bleach for 2 minutes and fixed in 10 ml of 150mM NaCl/0.05% Triton X-100 at 90°C for 10 seconds. After fixation, embryos were decanted into 40 ml of cold of 150mM NaCl/0.05% Triton X-100 for 5 minutes and then devitilinized in a 1:1 heptane:methanol solution. After washing in PBS/0.1% Triton X-100/0.2% BSA (PBSTB) embryos were incubated with a mouse monoclonal Dfz2 antibody (Nusse lab, Stanford Univ.) at a concentration of 1:10 in PBSTB/5% normal donkey serum overnight at 4°C. After washing in PBSTB, embryos were incubated with a 1:200 dilution (in PBSTB/5% normal donkey serum) of biotin conjugated anti-mouse secondary antibody (Jackson Immuno-chemicals, West Grove, PA) from a stock of 1.5 mg/ml in the manufacturer's suggested buffer, and incubated for 2 hours at room temp. Amplification of signal was obtained by adding horseradish peroxidase bound to Avidin and the signal was developed in DAB/H_2O_2 using Vector Labs (Burlingame, CA) ABC kit and protocol.

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IMAGE EVALUATION
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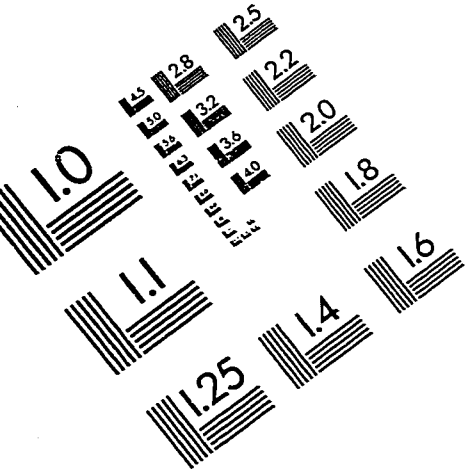

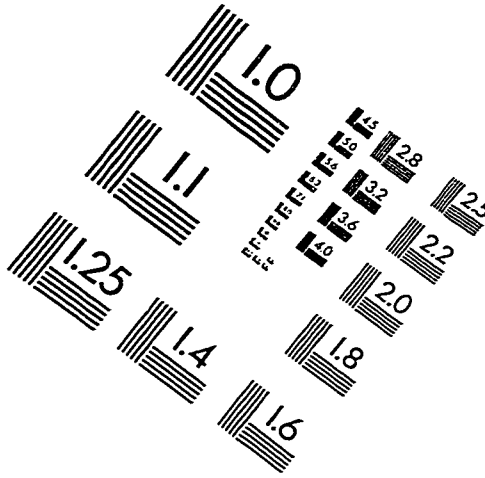

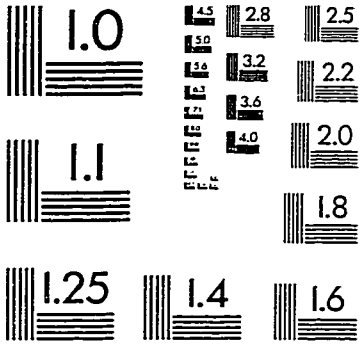

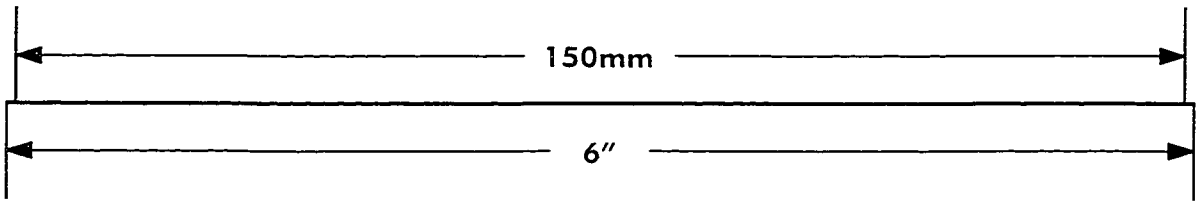

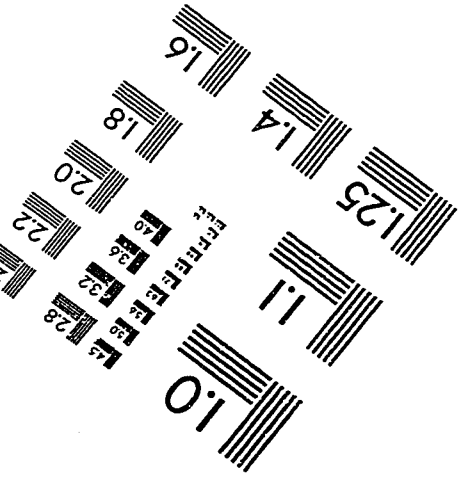

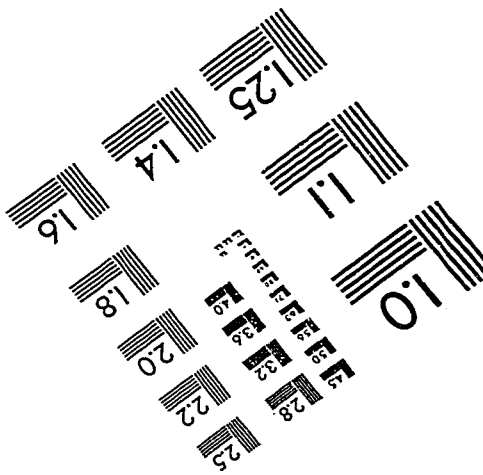

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