Frizzled and DFrizzled-2 function as redundant receptors for Wingless during *Drosophila* embryonic development

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SUMMARY

In cell culture assays, Frizzled and Dfrizzled2, two members of the Frizzled family of integral membrane proteins, are able to bind Wingless and transduce the Wingless signal. To address the role of these proteins in the intact organism and to explore the question of specificity of ligand-receptor interactions in vivo, we have conducted a genetic analysis of *frizzled* and *Dfrizzled2* in the embryo. These experiments utilize a small gamma-ray-induced deficiency that uncovers *Dfrizzled2*. Mutants lacking maternal *frizzled* and zygotic *frizzled* and *Dfrizzled2* exhibit defects in the embryonic epidermis, CNS, heart and midgut that are indistinguishable from those observed in *wingless* mutants. Epidermal patterning defects in the *frizzled, Dfrizzled2* double-mutant embryos can be rescued by ectopic expression of either gene. In *frizzled, Dfrizzled2* mutant embryos, ectopic production of Wingless does not detectably alter the epidermal patterning defect, but ectopic production of an activated form of Armadillo produces a naked cuticle phenotype indistinguishable from that produced by ectopic production of activated Armadillo in wild-type embryos. These experiments indicate that *frizzled* and *Dfrizzled2* function downstream of *wingless* and upstream of *armadillo*, consistent with their proposed roles as Wingless receptors. The lack of an effect on epidermal patterning of ectopic Wingless in a *frizzled, Dfrizzled2* double mutant argues against the existence of additional Wingless receptors in the embryo or a model in which Frizzled and Dfrizzled2 act simply to present the ligand to its bona fide receptor. These data lead to the conclusion that Frizzled and Dfrizzled2 function as redundant Wingless receptors in multiple embryonic tissues and that this role is accurately reflected in tissue culture experiments. The redundancy of Frizzled and Dfrizzled2 explains why Wingless receptors were not identified in earlier genetic screens for mutants defective in embryonic patterning.

Key words: Wingless, Frizzled, Wnt, *Drosophila*, Segmentation

INTRODUCTION

Wnts are secreted cysteine-rich glycoproteins with diverse roles in animal development (reviewed in Cadigan and Nusse, 1997). Frizzled proteins are cell surface Wnt receptors characterized by an extracellular cysteine-rich domain (CRD) followed by seven transmembrane segments (Vinson et al., 1989; Wang et al., 1996). Members of both protein families have been conserved in species as evolutionarily distant as nematodes and humans (Wang et al., 1996).

The prototypic member of the Frizzled family is the *Drosophila* tissue-polarity gene *frizzled* (*fz*), which was identified because of its phenotype in the adult cuticle (Adler et al., 1987; Gubb and Garcia, 1982). Null alleles of *fz* are viable as adults but their bristles, hairs and ommatidia lose their characteristic polarities (Adler et al., 1987; Gubb and Garcia, 1982; Vinson and Adler, 1987), a condition that is referred to

as a tissue or planar polarity phenotype. Historically, tissue polarity signaling has been studied without reference to Wnt signaling, although both signaling pathways are known to be affected by mutations in the *dishevelled* gene (Theisen et al., 1994; Krasnow et al., 1995). A second member of the Frizzled family in *Drosophila*, *Drosophila frizzled 2* (*Dfz2*), was identified by sequence homology (Bhanot et al., 1996). Both *fz* and *Dfz2* are expressed during embryonic and larval life (Adler et al., 1990; Bhanot et al., 1996), with *fz* expression in the embryo also being maternally derived (Park et al., 1994). Mutations affecting only the *Dfz2* gene have not been described.

The most intensively studied member of the Wnt family is the *Drosophila* segment polarity gene *wingless* (*wg*). *wg* has multiple essential functions during *Drosophila* development (reviewed in Klingensmith and Nusse, 1994). During embryogenesis, it is required for epidermal patterning

(Nüsslein-Volhard and Wieschaus, 1980), specification and formation of various neurons in the CNS (Chu-Lagraff and Doe, 1993), and morphogenesis of the midgut (Hoppler and Bienz, 1995) and heart (Park et al., 1996). During larval life, *wg* plays a critical role in patterning the wing (Couso et al., 1994), leg (Struhl and Basler, 1993) and eye imaginal discs (Treisman and Heberlein, 1998).

The Wg signal is transduced from the cell surface to the nucleus via the actions of Dishevelled (Dsh; Klingensmith et al., 1994) as noted above, Zeste-white 3 (Zw3), a serine-threonine kinase (Siegfried et al., 1994) and Armadillo (Arm), a β-catenin homolog (Peifer et al., 1994; reviewed in Cadigan and Nusse, 1997). Wg signalling stabilizes cytoplasmic Arm, which translocates to the nucleus where it forms a complex with dTCF/pangolin, an HMG-box protein (Brunner et al., 1997; Riese et al., 1997; van de Wetering et al., 1997). This complex then activates target genes (Brunner et al., 1997; Riese et al., 1997; van de Wetering et al., 1997). Work by several investigators has shown that these same components are utilized to transduce the Wg signal in different tissues or cell types but the identity of the Wg receptor(s) in these tissues had, until recently, been unknown.

Several lines of evidence indicate that the Fz family encodes receptors for the Wnt family of signaling molecules. In tissue-culture experiments, *Drosophila* S2 cells transfected with either *fz* or *Dfz2* bind exogenous Wg on the cell surface and transduce the Wg signal as assayed by the stabilization of Arm (Bhanot et al., 1996). Similar binding results have been obtained using Wg or *Xenopus* Wnt8 (XWnt8) and mammalian cells transfected with various Frizzled proteins (Hsieh et al., 1999). In *Xenopus* embryos, Xwnt-8 synergizes with rat Frizzled-1 (Rfz1) to activate transcription of the Wnt target, Siamois (Yang-Snyder et al., 1996) and Xwnt-5A synergizes with human Frizzled-5 (Hfz5) to induce axis duplication (He et al., 1997). Finally, experiments in *Drosophila* show that ectopic expression of *Dfz2* transgenes in wing imaginal discs expands the domain of Wg signaling and ectopic expression of the extracellular domain of fz or Dfz2 inhibits Wg signaling (Zhang and Carthew, 1998; Cadigan et al., 1998).

In addition to the cell culture and ectopic expression studies described above, loss-of-function mutations in *Caenorhabditis elegans* have also implicated particular Frizzled and Wnt proteins as potential receptor-ligand pairs. These experiments define the roles of *lin-17* (Fz) and *lin-44* (Wnt) in various asymmetric cell divisions (Herman et al., 1995; Sawa et al., 1996), *mom-5* (Fz) and *mom-2* (Wnt) in endoderm induction (Rocheleau et al., 1997), and *lin-17* and *egl-20* in neuronal migration (Harris et al., 1996; Maloof et al., 1999). However, in each of these cases, the phenotypes of the respective Wnt and Fz genes are qualitatively similar but are not identical, possibly due to additional factors (Sawa et al., 1996), lack of null mutations (Harris et al., 1996) or, in the case of *mom-5*, a possible role in antagonizing *mom-2* signaling (Rocheleau et al., 1997).

In this report, we focus on the role of Fz and Dfz2 in *Drosophila* embryogenesis. *fz* is widely expressed in the embryo and *Dfz2* is expressed in the CNS, midgut and dorsal vessel, and in a segmental pattern in the embryonic epidermis (Adler et al., 1990; Park et al., 1994; Bhanot et al., 1996). Our point of departure was the hypothesis, based on the tissue-culture experiments described above and the pattern of embryonic expression, that DFz2 and/or Fz might act in vivo as receptors for Wg. This model is superficially at odds with the observation that *fz* mutants do not display any defects in segment polarity as would be expected from a loss of Wg signaling during embryogenesis. If Fz functions as a Wg receptor during embryogenesis, its role must be redundant.

During the course of this study, several groups reported experiments that point to a requirement for both Fz and Dfz2 in Wg signalling in the embryo. These experiments used RNA interference (Kennerdell and Carthew, 1998) or large synthetic deficiencies (Bhat, 1998; Muller et al., 1999) to demonstrate that the simultaneous loss of both *fz* and *Dfz2* mimics the loss of *wg* on the embryonic epidermis and CNS. However, the low efficiency of the *wg*-like phenotype in the RNA interference experiments and the developmental arrest associated with elimination of approximately 5% of the genome in the synthetic deficiency experiments limit the conclusions that can be drawn from these experiments. Thus, a number of questions regarding the in vivo relationship between *wg*, *fz* and *Dfz2* remain to be addressed, in particular the question of whether loss of *fz* and *Dfz2* fully recapitulates the *wg* phenotype.

In the present study, we have generated a small deficiency that encompasses the *Dfz2* locus. We show that embryonic development in general, and Wg signaling in particular, is nearly normal in deletion homozygotes. However, embryos lacking both maternal *fz* and zygotic *fz* and *Dfz2* display defects in epidermal patterning, RP2 neuron specification, midgut morphogenesis and heart formation, which are extremely similar to the defects exhibited by *wg* mutants. We further show that the defects in epidermal patterning in the double mutants are rescued by ectopic expression of either *fz* or *Dfz2*, are unaffected by ectopic expression of *wg* and are over-ridden by ectopic expression of an activated form of *arm*. These results indicate that Dfz2 and Fz are the principal embryonic receptors for Wg and that they function redundantly in multiple tissues to transduce the Wg signal. Moreover, since the *fz, Dfz2* double mutants do not display defects in addition to those seen in *wg* mutants, it is likely that Wg serves as the predominant or only ligand for Dfz2 and Fz during embryogenesis.

MATERIALS AND METHODS

Cloning of *Dfz2* cDNA and genomic DNA

To isolate *Dfz2* cDNA clones, we screened a 0-9 hour embryo cDNA library (a gift from K. Zinn) with a PCR product corresponding to the first 237 nucleotides of the coding exon and sequenced three clones with the longest inserts 5′ of that point. To isolate genomic clones corresponding to exons 1 and 2, a *Drosophila* genomic library (a gift from T. Maniatis) was screened with a PCR product corresponding to exons 1 and 2. Sequences from the cDNA and genomic clones were compared to identify the splice sites between exons1/ 2 and exons2/ 3. Two P1 clones encompassing the *Dfz2* locus were obtained by PCR screening (Genome Systems) and the extended restriction map shown in Fig. 1 was assembled by construction of a lambda phage library from one of these P1 clones.

Drosophila stocks and recombinant chromosomes

The creation of the 469-2 deletion (hereafter referred to as *Df(3L)Dfz2*) and the various *fz* alleles used are described in the text. *Df(3L)Dfz2* was recombined with *fz* alleles in the following way. From a cross with *Df(3L)Dfz2* (at 76A) over a *Dichaete* (*D*), P[FRT^{2A}, *w$^+$*]

Table 1. Distribution of cuticle phenotypes seens with various *fz*, *Dfz2* chromosomes

Cross	Maternal	Paternal	Most severe genotype	n	Cuticle phenotype of unhatched embryos (%)					
					WT	1	2	3	4	5
A	$\frac{+}{+}$	$\frac{+}{+}$	$\frac{+}{+}$	75	100#	0	0	0	0	0
B	$\frac{Df(3L)Dfz2}{+}$	$\frac{Df(3L)Dfz2}{+}$	$\frac{Df(3L)Dfz2/+}{Df(3L)Dfz2/+}$	93	0	91	8	1	0	0
C	$\frac{fz^{R52},\ Df(3L)Dfz2}{fz^{D21},\quad +}$	$\frac{Df(3L)Dfz2}{+}$	$\frac{fz^{R52},\ Df(3L)Dfz2}{+,\quad Df(3L)Dfz2}$	103	0	84	16	0	0	0
D	$\frac{fz^{R52},\ +}{fz^{D21},\ +}$	$\frac{fz^{R52},\ Df(3L)Dfz2}{+,\qquad +}$	$\frac{fz^{R52},\ Df(3L)Dfz2}{fz^{D21},\qquad +}$	32	72	28	0	0	0	0
E	$\frac{fz^{R52},\ Df(3L)Dfz2}{+,\qquad +}$	$\frac{fz^{R52},\ Df(3L)Dfz2}{+,\qquad +}$	$\frac{fz^{R52},\ Df(3L)Dfz2}{fz^{R52},\ Df(3L)Dfz2}$	117	3	0	39	42	15	1
F	$\frac{fz^{R52},\ Df(3L)Dfz2}{+,\qquad +}$	$\frac{fz^{D21},\ Df(3L)Dfz2}{+,\qquad +}$	$\frac{fz^{R52},\ Df(3L)Dfz2}{fz^{D21},\ Df(3L)Dfz2}$	118	3	0	55	28	14	0
G	$\frac{fz^{D21},\ Df(3L)Dfz2}{+,\qquad +}$	$\frac{fz^{R52},\ Df(3L)Dfz2}{+,\qquad +}$	$\frac{fz^{R52},\ Df(3L)Dfz2}{fz^{D21},\ Df(3L)Dfz2}$	146	3	0	11	29	53	4
H	$\frac{fz^{R52},\ Df(3L)Dfz2}{fz^{D21},\qquad +}$	$\frac{fz^{R52},\ Df(3L)Dfz2}{+,\qquad +}$	$\frac{fz^{R52},\ Df(3L)Dfz2}{fz^{R52},\ Df(3L)Dfz2}$	86	5	0	0	0	0	95
I	$\frac{fz^{D21},\ Df(3L)Dfz2}{fz^{P21},\qquad +}$	$\frac{fz^{P21},\ Df(3L)Dfz2}{+,\qquad +}$	$\frac{fz^{D21},\ Df(3L)Dfz2}{fz^{P21},\ Df(3L)Dfz2}$	102	4	0	0	0	0	96

Cuticle preparations were made and analyzed as described in Materials and Methods. The classes of cuticle phenotype (1-5) are defined as follows: (1) trapezoidal arrays are slightly disorganized; distinct from wild type; phenotype seen in crosses B and C distinct from D. The n value for D is low because there are very few unhatched embryos. (2) Extra denticles observed in posterior compartment of at least two segments. (3) Denticles connecting at least 2 trapezoidal arrays. (4) Entire lawn of denticles. (5) Similar to *wg* mutants. See Fig. 2 for examples of these classes.
    #These are hatched larvae.

chromosome (*D* is at map position 70D1 and the w^+ at map position 79F), 20% of the w^+ chromosomes that had lost the *D* marker had acquired *Df(3L)Dfz2*. A chromosome containing *D*, *Df(3L)Dfz2*, P[FRT^{2A}, w^+] was then created by screening for w^+, *D* progeny and used to recombine various *fz* alleles (at map position 70D5) with *Df(3L)Dfz2*, P[FRT^{2A}, w^+] by selecting for the loss of *D*. The *wg* alleles used were wg^{CX4} and wg^{IN}. wg^{CX4} is a molecular null and wg^{IN} encodes a nonsecreted Wg protein (van den Heuvel et al., 1993).

Rescue and ectopic overexpression experiments

For the rescue experiments described in the text, where the *Da-Gal4* driver (Georgias et al., 1997) is used to drive either a *fz* (provided by P. Adler), *Dfz2* (Cadigan et al., 1998) *wg* (provided by I. Levine-Bar and H. Krause) or an activated *arm* (*arm*^{act}; Pai et al., 1997) transgene. P[*Da-Gal4*], *fz* recombinants were used to create P[*Da-Gal4*], *fz*, *Df(3L)Dfz2* P[FRT^{2A}, w^+] recombinants (P[*Da-Gal4*] and *fz* are closely linked). These could be crossed with P[*Da-Gal4*], *fz* flies to create the P[*Da-Gal4*], *fz*, *Df(3L)Dfz2* P[FRT^{2A}, w^+]/*Da-Gal4*, *fz* mothers for the experiments. For the ectopic *Dfz2* experiments, P[UAS-*Dfz2*$^{63-17}$ w^+] was recombined onto a *fz*, *Df(3L)Dfz2* P[FRT^{2A}, w^+] chromosome in the following way. Both P[w^+] minigenes have orange pigmentation when present in one copy, so recombinants containing both P[w^+] were identified. The presence of *fz* and *Df(3L)Dfz2* were then tested by complementation. For *UAS* lines of *fz*, *wg* and *arm*^{act} (all of which are on the second chromosome), stocks with these transgenes and *fz*^{R52}, *Df(3L)Dfz2* P[FRT^{2A}, w^+] were created over the 2-3 compound chromosome SM5a-TM6B. The compound chromosome ensured that the *UAS* chromosome and the *fz*, *Dfz2* recombinant co-segregates. Thus, one-quarter of the progeny will be *UAS-X*/+; P[*Da-Gal4*], *fz*, *Df(3L)Dfz2*/ *fz*, *Df(3L)Dfz2*.

An unforeseen problem with the crosses involving SM5-TM6

chromosomes is that a large number of poorly developed cuticles are observed (see Fig. 6D). Control crosses indicated that these occur in any cross with *fz*^{D21}, *Df(3L)Dfz2* mothers and SM5-TM6 fathers. It may be that the combination of the *fz*^{D21} and *Df(3L)Dfz2* deficiencies over this compound chromosome results in non-specific lethality of some (but not all) of these animals.

Cuticle preparation

Crosses were set up with the specific phenotypes indicated in Tables 1 and 2. Flies were allowed to lay eggs on grape juice plates for 20-24 hours. Flies were removed and the plates were incubated for an additional 24-36 hours at 25°C. During this time, moist yeast was placed in the center of each plate to attract hatching larvae. In this way, the majority of hatched animals could easily be removed. Unhatched eggs could then be removed with a wet brush and cuticles prepared as previously described (Cadigan et al., 1994), using a heptane/methanol treatment to remove the vitelline membrane.

In these experiments, there is usually a background of hatched wild-type larvae carried along in the cuticle preparation. As indicated in Tables 1 and 3, these individuals were typically between 1 and 8% of the total in crosses where one-quarter of the progeny did not hatch. In a few cases (crosses D in Table 1 and F in Table 3), the percentage of wild type is higher due to a lower number of unhatched larvae.

Immunohistochemistry

Antibody staining was performed as described (Reuter and Scott, 1990). Embryos were dechorionated, devitellinized in methanol/heptane and fixed in 4% formaldehyde in PBS. Antibody dilutions were as follows: monoclonal anti-Engrailed 4D9 at 1:1000 (Iowa Developmental Studies Hybridoma Bank; IDSHB), monoclonal anti-Eve skipped 3C10 at 1:20 (IDSHB), monoclonal anti-Wingless at

Table 2. The effect on RP2 specification of various mutant backgrounds

Embryonic genotype	% of embryos	Phenotype of affected embryos
Wild-type	0% (*n*=87)	All RP2 neurons present.
$\dfrac{Df(3L)Dfz2}{Df(3L)Dfz2}$	21% (*n*=53)	Either a missing or a displaced RP2 neuron in at least 1 hemisegment.
$\dfrac{fz^{R52},\,Df(3L)Dfz2}{fz^{P21},\,Df(3L)Dfz2}$	74% (*n*=46) 26% (*n*=46)	All RP2 neurons were missing. At least 1 hemisegment has a RP2 neuron.
$\dfrac{fz^{D21},\,Df(3L)Dfz2}{fz^{P21},\,Df(3L)Dfz2}$	100% (*n*=55)	All RP2 neurons are missing.

Immunostaining using mAb3C10 was performed as described in Materials and Methods. *Df(3L)Dfz2* / TM6GFP flies were self-crossed to produce *Df(3L)Dfz2* homozygous embryos, which were identified by their lack of staining with an anti-GFP antibody.

fz^{R52}, *Df(3L)Dfz2* / fz^{P21}, *Df(3L)Dfz2* embryos were produced by crossing fz^{R52}, *Df(3L)Dfz2*/ fz^{P21} / + females and fz^{P21}, *Df(3L)Dfz2* / + males. fz^{D21}, *Df(3L)Dfz2* / fz^{P21}, *Df(3L)Dfz2* embryos were produced by crossing fz^{D21}, *Df(3L)Dfz2* / fz^{P21} / + females and fz^{P21}, *Df(3L)Dfz2* / + males. fz^{R52}, *Df(3L)Dfz2* / fz^{P21}, *Df(3L)Dfz2* and fz^{D21}, *Df(3L)Dfz2* / fz^{P21}, *Df(3L)Dfz2* embryos were recognized by their characteristic small size compared to normal embryos.

1:10 (a gift from S. Cohen), rat anti-Titin at 1:10000 (a gift from C. Machado), rat anti-Teashirt (a gift from M. P. Scott) at 1:2000, monoclonal anti-lab at 1:1000 (a gift from D. Andrew), and polyclonal anti-GFP (Clontech) at 1:200. Biotinylated secondary antibodies (at 1:500 except for anti-Titin immunostaining which was at 1:1000) and ExtrAvidin Peroxidase conjugate (from Sigma at 1:200 except for anti-Titin immunostaining which was at 1:500) were used to develop the diaminobenzidine and nickel chloride color reactions. For double staining with anti-Eve and anti-GFP, the anti-Eve reaction was developed using a biotinylated secondary antibody and DAB while the anti-GFP reaction was developed using an alkaline phosphatase-conjugated secondary antibody. The alkaline phosphatase reaction was developed using 5-bromo-4-chloro-3-indolyl phosphate (Boehringer)

and nitroblue tetrazolium (Boehringer). Embryos were cleared and mounted in methyl salicylate.

RESULTS

Dfz2 gene structure and identification of a P-element within the *Dfz2* transcription unit

The *Dfz2* gene has previously been mapped to 76A (Bhanot et al., 1996). Fig. 1 shows a restriction map of the *Dfz2* locus together with the structure of the *Dfz2* transcript deduced from an analysis of six cDNA clones derived from a *Drosophila* embryo library. The *Dfz2* gene consists of at least three exons, with the entire open reading frame being contained on a single exon, exon 3. We note that the full-length *Dfz2* transcript may contain noncoding sequences in addition to those indicated in Fig. 1 since the size of the transcript predicted from the cDNA clones isolated thus far is about 4 kb while northern blotting reveals a transcript of about 5.5 kb (Bhanot et al., 1996).

To identify a P-element integrated within or near the *Dfz2* locus, we characterized five *Drosophila* lines in which a P-element had been reported to map in or near 76A. Genomic DNA flanking each P-element insertion site was cloned by plasmid rescue and hybridized against a panel of P1 clones carrying the *Dfz2* locus. The insertion site of a single P-element line, 469, was found to reside on a subset of the P1 clones, and further mapping and sequencing showed that the 469 P-element is inserted 60 bases upstream of *Dfz2* exon 1 (Fig. 1). *Dfz2* transcripts from 469 embryos are indistinguishable from the wild type in size but are reduced in abundance (data not shown). Animals homozygous for this insertion show no obvious morphologic defects during embryogenesis or adulthood.

Deletion of the *Dfz2* locus and construction of *fz, Dfz2* double mutants

The 469 line was used in a gamma-ray mutagenesis screen

Table 3. Effect on ubiquitous expression of wg or an activated form of arm on the *fz, Dfz2* segment polarity phenotype

Cross	$\dfrac{DaGal4,\,fz^{D21},\,Df(3L)Dfz2}{DaGal4,\,fz^{R52},\,+}$ mothers crossed to:	*n*	None	Weak	Strong	Naked	'Ghost'
			Segment Polarity Phenotype of unhatched embryos (%)				
A	$\dfrac{fz^{R52},\,Df(3L)Dfz2}{TM6}$	113	8	0	91	0	1
B	$\dfrac{UAS\text{-}Dfz2,\,fz^{R52},\,Df(3L)Dfz2}{TM6}$	117	5	94	1	0	0
C	$\dfrac{UAS\text{-}fz;\,fz^{R52},\,Df(3L)Dfz2}{SM5a\text{-}TM6B}$	190	2	59	0	0	39
D	$\dfrac{UAS\text{-}wg;\,fz^{R52},\,Df(3L)Dfz2}{SM5a\text{-}TM6B}$	318	2	0	26	51	21
E	$\dfrac{UAS\text{-}arm^{act};\,fz^{R52},\,Df(3L)Dfz2}{SM5a\text{-}TM6B}$	150	1	0	0	79	20
F	$\dfrac{+,\qquad +}{SM5a\text{-}TM6B}$	123	26	0	0	0	74

Cuticle preparations were made and analyzed as described in Materials and Methods. The genotype of the stocks used were confirmed in the following controlled crosses (data not shown). When the P[*DaGal4*], *fz, Dfz2*/+ mothers were crossed to *UAS-wg* or *UAS-arm^{act}*, 100% of the unhatched embryos had the expected naked cuticle. When the fathers in crosses B-E were crossed to *fz/fz, Dfz2*/+ mothers without the DaGal4 transgene, the large majority (>85%) of all unhatched embryos had a strong segment polarity phenotype. See Figs 6 and 7 for micrographs of the respective phenotypes.

Fig. 1. Structure of the *Dfz2* locus and the position of the 469 P-element. A restriction map is shown for a 70 kb region of genomic DNA encompassed by the overlapping lambda phage clones shown at the bottom. The *Dfz2* transcription unit is shown at the center. The transcript structure was deduced from the structure of three cDNA clones and may not represent the complete transcript. The 469 P-element integration site is located approximately 60 bp 5′ of the genomic region corresponding to the 5′-end of the longest cDNA clone. The possible existence of additional transcription units in this region has not been investigated. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sal*I.

(4000 rad) to generate deficiencies in the *Dfz2* locus, and one mutant line, 469-2, was found to harbor a deletion that encompasses 75F10-11to 76A1-5 as judged by cytological mapping of its polytene chromosomes. Complementation tests indicated that this deletion did not uncover naked (75F6) a segment polarity gene that affects Wg targets (Dougan and Dinardo, 1992; Bejsovec and Wieschaus, 1993). In situ hybridization with a Dfz2 probe on polytenes or 469-2

homozygous embryos indicated that the Dfz2 locus and expression were absent (data not shown). For convenience, we will simply refer to the 469-2 deletion hereafter as *Df(3L)Dfz2* or as a *Dfz2* deficiency.

As described below, our initial assessment of the mutant phenotype focused on patterning in the embryonic cuticle. In wild-type larvae, the cuticle is covered by an alternating pattern of denticle belts and naked cuticle that is specified by the interaction of segment polarity genes in the embryonic epidermis. Wg action specifies both denticle diversity in the anterior half of each segment and naked cuticle in the posterior half. In *wg* mutants, there is loss of naked cuticle characteristic of the posterior half and reduction in the diversity of denticles covering the anterior half of each segment such that the denticles present on the cuticle are morphologically similar to those of wild-type row 5 (Fig. 2G,K; Dougan and Dinardo, 1992; Bejsovec and Wieschaus, 1993).

Dfz2-deficiency homozygotes die shortly after hatching and exhibit a subtle disorganization of denticle patterning with occasional ectopic denticles in posterior compartments. These data suggest that *Dfz2* and/or other genes removed by the 469-2 deficiency play a minor or largely redundant role in cuticle patterning during embryogenesis. In light of the similarity in sequence and Wg-binding properties exhibited by Fz and Dfz2, we tested the hypothesis that Fz and Dfz2 might function redundantly by constructing double mutants in which various *fz* alleles (at map position 70D5) were recombined with the *Dfz2* deficiency. For these experiments,

Fig. 2. Loss of *fz* and *Dfz2* function results in *wg*-like cuticle phenotypes. All pictures show the ventral side of the embryo with anterior at the top.
(A-E) Abdominal segments 1 through 7 or 8. (A) Wild-type cuticle. Trapezoidal arrays of denticles in the anterior part of each segment are interspersed with regions of naked cuticle.
(B,C) Zygotic *fz^{R52}, Dfz2*-deficiency homozygotes that are typical of class 2 and 3 in Table 1 respectively. (B) The arrows point to the extra denticles seen in the posterior part of the segments.
(C) Near complete replacement of naked cuticle with denticles in one segment. (D) Zygotic *fz^{D21}, Dfz2/fz^{R52}, Dfz2* embryo typical of class 4 from Table 1. The naked cuticle in each abdominal segment has been replaced with denticles.
(E) Cuticle from *Dfz2*-deficiency homozygote. This animal is subtly different from wild type (characterized as class 1 in Table

fz, Dfz2 (zygotic only)

1), but overall retains the denticle/naked cuticle polarity of each segment. (F) Embryo completely lacking maternal and zygotic *fz* and zygotic *Dfz2* (*fz^{D21}, Dfz2/fz^{P21}, Dfz2*). This animal exhibits no naked cuticle, a severe reduction in size and head defects (class 5 in Table 1). Segmental periodicity of the denticles is still apparent. (G) A *wg* null (*wg^{IN}/wg^{IN}*) embryo appears very similar to the *fz, Dfz2* mutant shown in F. A different *wg* null allele (*wg^{CX4}*) gave identical results. (H) Embryo null for *hh*. These embryos also have lost their ventral naked cuticle, but have a more severe disruption of segmentation than *wg* or the *fz, Dfz2* mutants (F). (I-K) Higher magnification phase micrographs of wild-type (I), and *fz, Dfz2* (J) and *wg* (K) cuticles. In cuticles from *wg* and *fz, Dfz2* embryos there is a similar transformation of denticles to the type characteristic of row 5 (indicated by arrows in panel I).
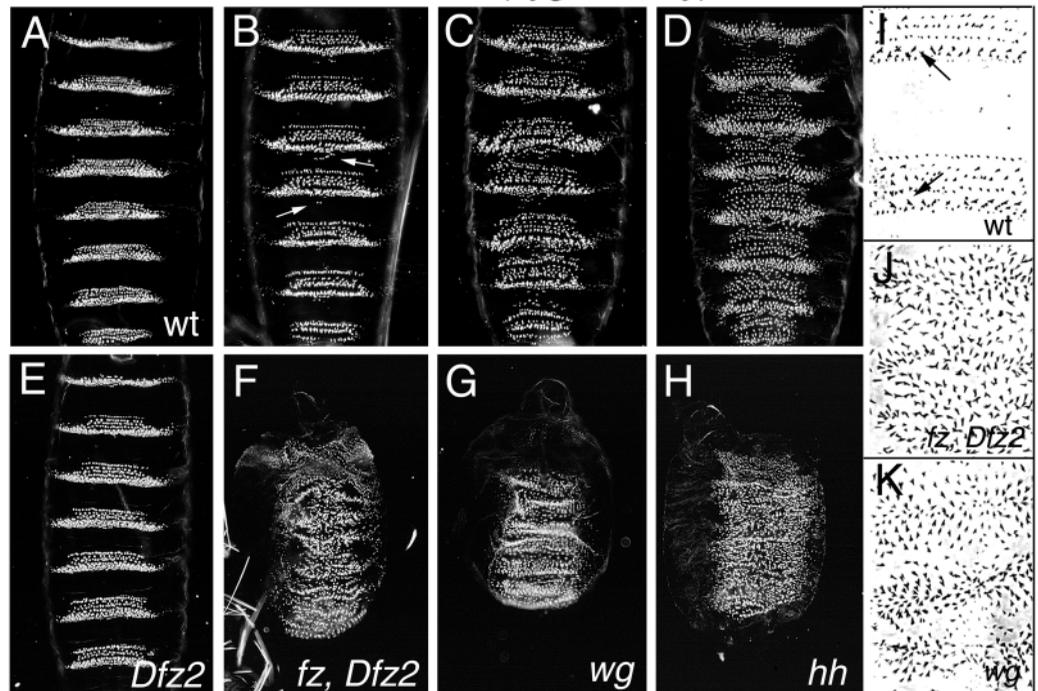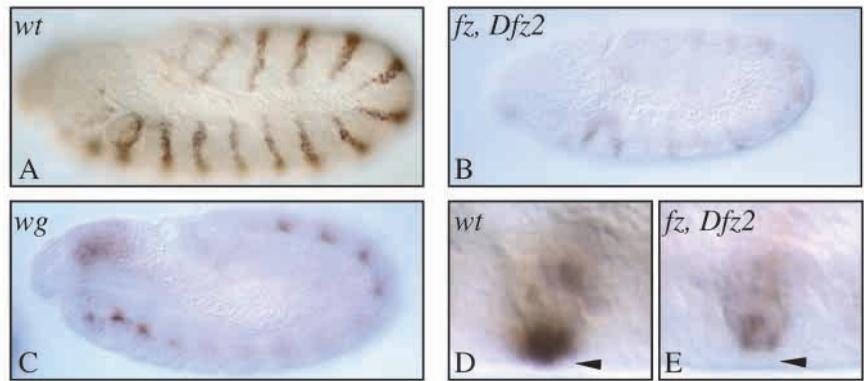
anti-En

anti-Wg

Fig. 3. En and Wg expression is lost in *fz, Dfz2* mutant embryos. Lateral views of stage 10 embryos stained with (A-E) anti-En and (F,G) anti-Wg antibodies. (A,D,F) Wild-type embryos. (B,E,G) *fz, Dfz2* mutant embryos obtained from a cross between *fz^{D21}, Df(3L)Dfz2/fz^{P21}*, + mothers and *fz^{P21}*, Df(3L)Dfz2/TM6 fathers. (C) *wg^{IN}* mutant embryo. (D,E) High-magnification views of single segments. Wild-type embryos (A,D) show En expression in both the CNS and epidermis (arrowhead in D) whereas *fz, Dfz2* (B,E) and *wg* embryos (C) show a loss of En expression in the epidermis (arrowhead in E) but not in the CNS where En expression is independent of Wg signaling. In *fz, Dfz2* embryos, the stripes of Wg staining (G) are both narrower and reduced in intensity compared to wild type (F).
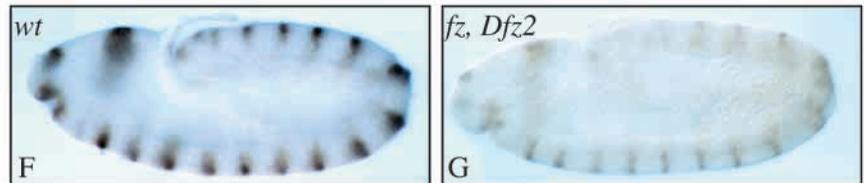

Fig. 4. Defects in RP2 specification and heart morphogenesis in *fz, Dfz2* mutant embryos. (A-E) Ventral views of embryos stained with anti-Eve to visualize RP2 neurons. Arrows mark RP2 neurons; arrowheads mark the positions of missing RP2 neurons. (A) Wild-type embryo. (B) *fz, Dfz2* mutant embryos from a cross between *fz^{D21}, Df(3L)Dfz2/fz^{P21}*, + mothers and *fz^{P21}, Df(3L)Dfz2/*TM6C fathers. RP2 neurons are completely missing. (C) *fz, Dfz2* embryos from a cross between *fz^{R52}, Df(3L)Dfz2/fz^{P21}*, + mothers and *fz^{R52}, Df(3L)Dfz2/*TM6 fathers show a variable loss of RP2 neurons. (D) *wg* null embryos (*wg^{IN}/wg^{IN}*) show a complete loss of RP2 neurons. (E) *Df(3L)Dfz2/Df(3L)Dfz2* embryos show occasional loss or misplacement of RP2 neurons. See Table 2 for quantitation of the RP2 loss in the different genetic backgrounds. These embryos were derived from a *Df(3L)Dfz2/*GFP-TM6 stock and identified by their lack of GFP immunostaining. (F-I) Lateral views of embryos stained with anti-Eve to visualize cardiac precursors. (F) Wild-type embryos showing Eve-stained cardiac precursors. (G) *fz, Dfz2* embryo from a cross between *fz^{D21}, Df(3L)Dfz2/fz^{P21}*, + mothers and *fz^{P21}, Df(3L)Dfz2/*TM6C fathers. (H) *fz, Dfz2* mutant embryo from a cross between *fz^{R52}, Df(3L)Dfz2/fz^{P21}*, + mothers and *fz^{R52}, Df(3L)Dfz2/*TM6I fathers. (I) *wg* null embryos (*wg^{IN}/wg^{IN}*). (G-I) Mutant embryos show a complete loss of Eve-stained cardiac precursors. (G) The plane of focus reveals expression of Eve in the CNS. Embryos are stages 16 (A,E), 14 (B-D) and 12 (F-I).
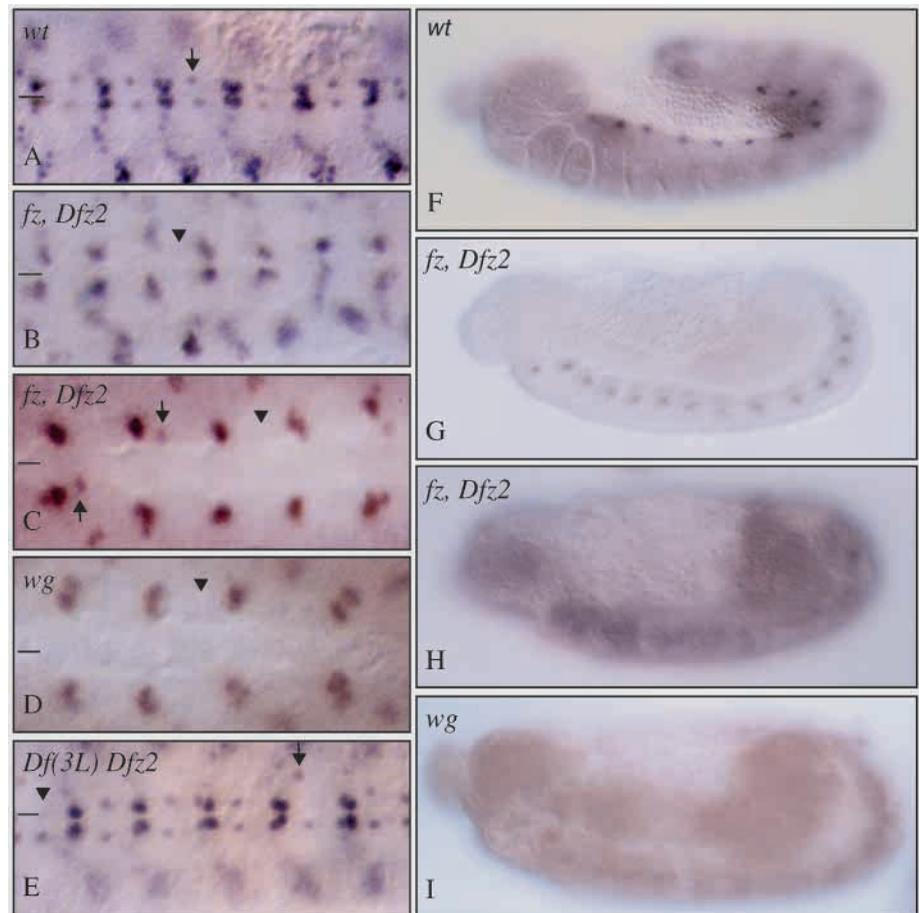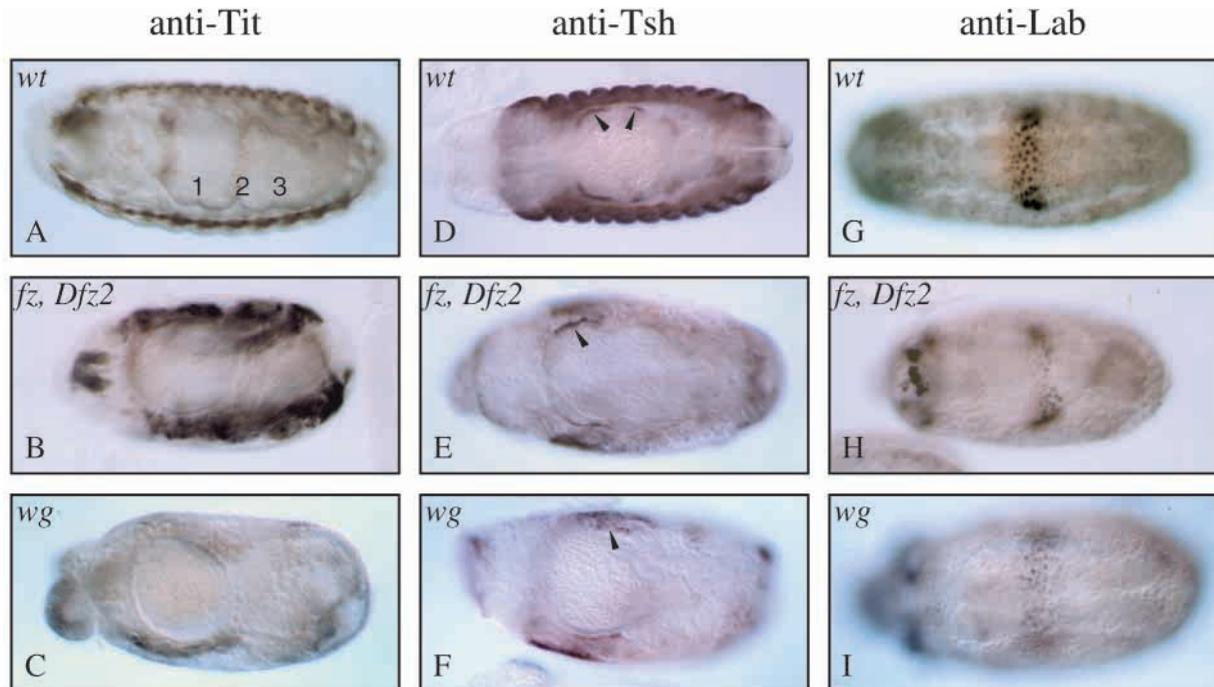
Fig. 5. Defects in midgut development in *fz*, *Dfz2* mutant embryos. (A,D,G) Wild-type embryos; (B,E,H) *fz*, *Dfz2* mutant embryos from a cross between *fz^D21*, Df(3L)*Dfz2/fz^P21*, + mothers and *fz^P21*, Df(3l)*Dfz2*/TM6C fathers; (C,F,I) *wg* null embryos (*wg^IN/wg^IN*). (A-C) Lateral views of embryos stained with anti-Titin to visualize midgut morphology. (A) Wild-type embryos have three midgut constrictions while (B) *fz*, *Dfz2* and (C) *wg* embryos have only one constriction. Disorganization of body wall muscles is evident in B and C. (D-F) Dorsal views of embryos stained with an anti-Tsh antibody. (D) Wild-type embryos express Tsh in two domains (marked by arrowheads) at stage 14. Tsh expression is lost in the posterior domain in (E) *fz*, *Dfz2* and (F) *wg* embryos. (E,F) Arrowheads mark the anterior domain of Tsh expression. (G-I) Dorsal views of embryos stained with an anti-Lab antibody. Wild-type embryos (G) express Lab at high levels in a stripe in the endoderm while in *fz*, *Dfz2* (H) and *wg* (I) mutants, expression of Lab is significantly reduced.

and in those described below, we have used the following *fz* alleles (Jones et al., 1996): (1) *fz^P21*, a frameshift near the amino terminus that behaves as a null mutation, (2) *fz^R52*, a premature termination codon that removes the last transmembrane domain and which behaves as a null mutation with respect to the adult tissue polarity phenotype, but which behaves as a hypomorph in the embryo experiments described below, and (3) *fz^D21*, a deficiency that encompasses polytene bands 70D2-70E8. The *fz^R52* allele produces small amounts of a truncated Fz protein as determined by western blotting while *fz^D21* and *fz^P21* produce no detectable protein (Jones et al., 1996).

While embryos homozygous for the *Dfz2* deficiency have essentially normal segmentation (Fig. 2E), *Dfz2*-deficiency embryos in which both *fz* alleles are also mutant display variable segmentation defects (Fig. 2B-D). The defects range from a few extra denticles in the posterior part of some segments (Fig. 2B) to a complete replacement of naked cuticle with denticles on the ventral side of the embryo (Fig. 2D; see Table 1 for quantitation of the frequencies). This "lawn of denticles" is reminiscent of phenotypes obtained with a temperature-sensitive allele of *wg* (Bejsovec and Wieschaus, 1993) or mutations in DTCF, the DNA-binding protein that mediates Wg signaling in the nucleus (Brunner et al., 1997; van de Wetering et al., 1997). Thus, the absence of zygotic *fz* and *Dfz2* produces a cuticle phenotype consistent with a partial defect in Wg signaling.

The role of maternal and zygotic *fz* and *Dfz2* in patterning of the embryonic cuticle

While the expression of *Dfz2* appears to be predominantly zygotic (Bhanot et al., 1996), the high levels of *fz* transcripts found in early embryos (Park et al., 1994) suggests that it has significant maternal expression. Because *fz* mutants are viable, maternal contribution can easily be removed by using *fz*, *Dfz2/fz*, + mothers (crosses H and I in Table 1). Crossing these mothers with *fz*, *Dfz2/+*, + fathers results in embryos in which naked cuticle fate is lost, and the cuticle is covered with denticles (Fig. 2F) that predominantly resemble those found in row 5 in the normal cuticle (Fig. 2I). This phenotype is very similar to that of *wg* null mutants (Fig. 2G,K) and distinct from that of *hedgehog* (*Hh*) mutants (Fig. 2H). The difference in the phenotypes between crosses E-G and H, I in Table 1 indicate that one copy of maternal *fz* can partially rescue the segmentation defect.

Crosses C and D were designed to test whether either zygotic *fz* or *Dfz2* could rescue the phenotype. In cross C, one quarter of the embryos lack maternal *fz* and zygotic *Dfz2*, but have one copy of zygotic *fz*. Most of the unhatched embryos from this cross showed the subtle denticle defect seen in *Dfz2*-deficiency homozygotes, and a few have very weak segment polarity defects. This indicates that one zygotic copy of *fz* can almost completely rescue the segment polarity defect. In cross D, where one half of the embryos are mutant for maternal and zygotic *fz* and heterozygous for *Dfz2*, most embryos hatch and

the few that do not have either wild-type cuticles or only subtle denticle defects. These data indicate the following order of rescuing activity: zygotic *Dfz2*>zygotic *fz*>maternal *fz*.

This cuticle analysis also indicates that the *fz^{R52}* allele is not null for the segmentation defects observed. For example *fz^{R52}*, *Dfz2/fz^{R52}*, *Dfz2* embryos (cross E in Table 1) have less severe defects (81% have cuticles similar to those in Fig. 2B or C) compared with *fz^{D21}*, *Dfz2/fz^{R52}*, *Dfz2* embryos (cross G in Table 1; 57% have cuticles similar to those in Fig. 2D or F). Also, subtle phenotypic differences can be seen in the density and type of denticle observed in cuticles lacking maternal *fz* as well as zygotic *fz* and *Dfz2*, with *fz^{P21}/fz^{D21}* being more severe than *fz^{R52}/fz^{R52}* (data not shown). The hypomorphic nature of the *fz$R52$* allele for Wg signaling is confirmed by the immunostaining experiments described below.

In the text that follows, embryos lacking maternal *fz* and zygotic *fz* and *Dfz2* will simply be referred to as *fz, Dfz2* double mutants. The exact allele combinations are noted in the text, tables and figure legends. In each of the experiments described below in which *fz, Dfz2* double mutants were characterized morphologically or by immunostaining, approximately 25% of the embryos showed an aberrant phenotype as expected. Since the control crosses in Table 1 indicate that the presence of one copy of *fz* or *Dfz2* suffices to prevent severe defects in embryogenesis, we presume that in each cross the approximately one quarter of embryos that are aberrant represent the *fz, Dfz2* mutant embryos. For immunostaining analyses performed late in embryonic development, the *fz, Dfz2* embryos were readily distinguished by their decreased length.

fz and *Dfz2* are required during embryogenesis to maintain *en* and *wg* expression in the epidermis

In the wild-type epidermis, *wg* functions in an autocrine pathway to maintain its own expression (Hooper, 1994; Yoffe et al., 1995) and in a paracrine regulatory loop to maintain expression of *en* in adjacent cells (DiNardo et al., 1988; Martinez-Arias et al., 1988). In the epidermis at gastrulation, when *wg* function is first detected, a stripe of cells in the anterior half of each parasegment expresses *wg* and an adjacent stripe of cells in the posterior half express *en* (DiNardo et al., 1988; Martinez-Arias et al., 1988). This pattern is initiated by pair-rule and gap genes, but its maintenance requires paracrine signaling by Wg to the *en* expressing cells and both paracrine signaling by Hh and autocrine signaling by Wg to the *wg* expressing cells. Thus, in *wg* mutant embryos the pattern of *wg* and *en* gene expression is initiated correctly but is not maintained.

In *fz, Dfz2* double-mutant embryos, the En stripes begin to fade at stage 9/10 and are completely absent from the epidermis by mid stage 10 (Fig. 3B) similar to *wg* mutants (Fig. 3C). By contrast, *en* expression within the CNS is maintained (Fig. 3E) as it is in *wg* mutants (data not shown). Consistent with a defect in Wg signaling, Wg expression is greatly reduced in fz, Dfz2 mutants (Fig. 3G). The effect on En and Wg expression in the epidermis is less severe in *fz, Dfz2* double-mutant embryos heterozygous for *fz^{R52}/fz^{D21}* compared to embryos carrying *fz^{D21}/fz^{P21}* (data not shown).

fz and *Dfz2* are required for the specification of RP2 neurons and heart precursors

At the end of gastrulation, *wg* participates in the

morphogenesis of various embryonic structures. In the embryonic central nervous system, *wg* is expressed by row 5 neuroblasts (NBs) and its function is required to specify NBs in rows 4 and 6 (Chu-Lagraff and Doe, 1993). Null mutants of *wg* show a loss or duplication of several NBs, the most extensively studied being NB-4. The NB-4 lineage gives rise to two RP2 motoneurons per segment that innervate the dorsal musculature and are missing in *wg* mutant embryos (Chu-Lagraff and Doe, 1993).

RP2 neurons are marked by their expression of *even-skipped* (*eve*; Fig. 4A,B). Mutant embryos missing maternal *fz* and zygotic *fz* and *Dfz2* or missing only zygotic *Dfz2* were examined using an antibody against Eve. *fz, Dfz2* double-mutant embryos carrying the *fz^{D21}/fz^{P21}* alleles show a complete loss of RP2 neurons in all hemisegments. As observed in the epidermis, the *fz^{R52}* allele shows residual activity: in *fz, Dfz2* double mutants carrying the *fz^{R52}* allele, approximately 26% of the double-mutant embryos showed Eve-positive RP2 staining in 1-3 hemisegments. Interestingly, 469-2 homozygous embryos also show a weakly penetrant RP2 phenotype. In approximately 21% of the 469-2 homozygous embryos, an RP2 neuron is either missing or misplaced in 1-3 hemisegments (see Table 2 for details). We conclude that *fz* and *Dfz2* are largely but not entirely redundant in specifying RP2 identity. These observations are in good agreement with those reported by Bhat (1998).

Wg signaling is required to specify cardiac precursor cells by directly or indirectly maintaining the expression of the homeobox gene *tinman* (*tin*) in the cardiac mesoderm (Park et al., 1996). *eve* is expressed in a subset of heart precursor cells at stage 10/11, and these cells are missing in *wg* mutants (Lawrence et al., 1995; Fig. 4I). In *fz, Dfz2* double-mutant embryos, *eve*-expressing cardiac precursors are similarly missing (Fig. 4G,H), a phenotype that is completely penetrant even in *fz, Dfz2* double mutants carrying *fz^{R52}* (Fig. 4H).

## *fz* and *Dfz2* are required for midgut development

In the wild-type embryo, Wg signaling is required independently in both germ layers that constitute the midgut. It is required in the visceral mesoderm for the formation of the second midgut constriction (Immergluck et al., 1990) and in the midgut endoderm (Hoppler and Bienz, 1995). At the molecular level, Wg activates the expression of the zinc-finger gene *Teashirt* (*tsh*) in the visceral mesoderm (Mathies et al., 1994; Fig. 5D) and the homeotic gene *Labial* (*lab*) in the endoderm (Immergluck et al., 1990; Reuter et al., 1990; Yu et al., 1996; Fig. 5G). In *wg* embryos, *Tsh* expression is lost from the central midgut while its anterior expression is maintained (Fig. 5F). In the endoderm of *wg* embryos, the stripe of *lab* expression is not induced to the wild-type level (Fig. 5I).

We have characterized midgut development in *fz, Dfz2* double-mutant embryos using three markers. Titin, a protein expressed in somatic and visceral muscles (Bilder and Scott, 1998) was used to visualize midgut morphology (Fig. 5A-C), and Tsh (Fig. 5D-F) and Lab (Fig. 5G-I) were used as markers for Wg signaling in the visceral mesoderm and endoderm, respectively. *fz, Dfz2* embryos lack the normal number of midgut constrictions (Fig. 5B). These embryos are also missing *tsh* expression in the central domain although its anterior expression persists (Fig. 5E). Finally, *lab* expression is

Fig. 6. Rescue of the *fz, Dfz2* cuticle phenotype by ectopic expression of either *fz* or *Dfz2*. Micrographs are in the same orientation as in Fig. 2. Embryos in all panels are the progeny of P[*DaGal4*], *fz*^D21^, Df(3L)Dfz2/P[*DaGal4*], *fz*^R52^ mothers and fathers of the following genotypes. (A) *fz*^R52^, Df(3L)Dfz2/TM6B fathers produce *fz, Dfz2* mutant embryos that show the characteristic *wg*-like cuticle; (B) P[*UAS-Dfz2*], *fz*^R52^, Df(3L)Dfz2/TM6B fathers produce *fz, Dfz2* mutant embryos that are rescued to an almost wild-type phenotype (arrows point to occasional extra denticles); (C) P[*UAS-fz*]; *fz*^R52^, Df(3L)Dfz2/SM5a-TM6B fathers produce

unhatched embryos that also have a few extra denticles or that have "ghost" cuticles as shown in D. These "ghosts" were also seen in a control cross with +, +/SM5a-TM6B and are presumably *fz*^D21^, Df(3L)Dfz2/SM5a-TM6B animals that fail to develop. See Materials and Methods and Table 3 for additional details.
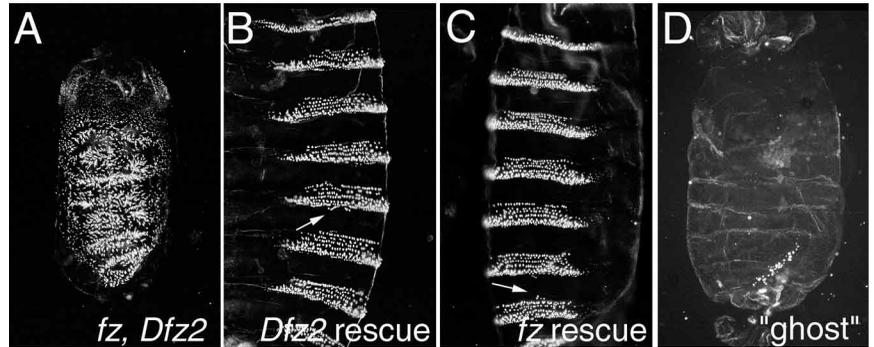
significantly reduced (Fig. 5H). By each of these criteria, the phenotype produced by the combined loss of *fz* and *Dfz2* resembles that produced by loss of *wg* (Fig. 5C,F,I)

Ectopic expression of *fz* or *Dfz2* rescues defects in segment polarity observed in double-mutant embryos

Since the 469-2 deficiency removes a number of genes in the 76A interval, it is possible that the defects described above are due to the loss of genes other than *Dfz2*. To address this possibility, we asked whether a *Dfz2* transgene (*UAS-Dfz2*) under the control of a *Daughterless GAL4* (*Da-GAL4*; Georgias et al., 1997) driver could rescue the *fz, Dfz2* double-mutant cuticle phenotype. In this experiment, we observe that ectopic expression of *Dfz2* produces nearly complete rescue (Fig. 6B), strongly suggesting that among the genes deleted in the 469-2 interval, *Dfz2* is the only one that is relevant to the segment polarity and cuticle patterning defects observed in *fz, Dfz2* double mutants. We note that this experiment does not eliminate the formal possibility that a third as-yet-undiscovered *frizzled*-like gene might reside in the 469-2 interval and play a redundant role in Wg signaling. Near complete rescue of the *fz, Dfz2* cuticle phenotype is also achieved by ectopic expression of a *fz* transgene (Fig. 6C), the expected result given that the *fz*^P21^ and *fz*^R52^ are point mutations within the *fz* coding region. The ability of either ectopic *fz* or *Dfz2* to rescue the segment polarity defects in the *fz, Dfz2* double mutants further confirms the overlap in function of these two proteins in the embryonic epidermis.

Effect of ectopic *wg* and activated *arm* on the *fz, Dfz2* double-mutant phenotype

Although the data presented thus far are most readily explained by a model in which Fz and Dfz2 act as signal transducing receptors for Wg, it is formally possible they are not bone fide Wg receptors but rather act to concentrate, transport or stabilize Wg, or present Wg to its real receptor. Proteoglycans are presumed to act in this manner and mutations affecting proteoglycan synthesis produce *wg*-like phenotypes in the embryo (Binari et al., 1997; Hacker et al., 1997; Haerry et al., 1997) that can be rescued by high level ectopic expression of *wg* (Hacker et al., 1997). Moreover, in light of the diverse actions of Wg during embryonic development, it is also possible that Wg utilizes additional and as yet uncharacterized

receptors and/or coreceptors that act independently or in conjunction with Fz and Dfz2. Under any of the preceding scenarios, high level ectopic expression of *wg* might either modify or correct the patterning defects seen in *fz, Dfz2* double-mutant embryos, as was observed for the proteoglycan biosynthetic mutants. We therefore tested this possibility by overexpressing *wg* in the *fz, Dfz2* double-mutant embryos using a *Da-GAL4* driver. In this experiment, no alterations in the *fz, Dfz2* cuticle phenotype were observed, arguing against the existence of additional *wg* receptors in the segment polarity pathway and against models in which Fz or Dfz2 concentrate, stabilize or transport Wg or present it to a coreceptor.

If Fz and Dfz2 act as Wg receptors, then constitutive activation of any of the intracellular components of the Wg signaling cascade should bypass the receptor defect in *fz, Dfz2* double-mutant embryos. To test this hypothesis, we determined the phenotypic effect of ectopic expression of a constitutively activated *arm* transgene (Pai et al., 1997) in the *fz, Dfz2* double-mutant background. As seen in Fig. 7C and D, the naked cuticle phenotype is produced in both wild-type and *fz, Dfz2* double-mutant embryos expressing the activated *arm*. Taken together, the *wg* and *arm* ectopic expression experiments indicate that Fz and Dfz2 function downstream of Wg and upstream of Arm, consistent with their proposed roles as Wg receptors.

DISCUSSION

Dfz2 and Fz are redundant receptors for Wg in multiple embryonic tissues

The results presented here strongly suggest that Fz and Dfz2 are the principal Wg receptors in the embryo. The evidence for this claim is two fold. First, mutants lacking maternal *fz* and zygotic *fz* and *Dfz2* exhibit defects in the embryonic epidermis, CNS, heart and midgut that are indistinguishable from those observed in *wg* mutants. Second, overexpression of *wg* does not detectably alter the patterning defects of the double-mutant embryos. The redundancy of Fz and Dfz2 explains why Wg receptors were not identified in earlier genetic screens for mutants defective in embryonic patterning (Nüsslein-Volhard and Wieschaus, 1980; Perrimon et al., 1996).

In the fz, Dfz2 mutant analysis, defects identical to *wg* were

Fig. 7. Effect of ectopic *wg* and activated *arm* expression on *fz, Dfz2* embryos. Micrographs are in the same orientation as in Fig. 2. (A,B) *DaGal4* driving *UAS-wg* in wild-type (A) or *fz, Dfz2* (B) embryos. The characteristic lack of denticles is observed when *wg* is ubiquitously expressed. However, ubiquitous expression of *wg* has no effect on *fz, Dfz2* cuticles. (C,D) *DaGal4* driving *UAS-arm^act* in wild-type (C) or *fz, Dfz2* (D) embryos. In both cases, nearly all cuticles from unhatched embryos had the naked cuticle phenotype shown (except for the "ghost" cuticles caused by the SM5a-TM6B balancer). Note that in D, we cannot distinguish individuals that are heterozygotes from *fz, Dfz2* mutants, since all embryos in this cross have the naked cuticle phenotype and are indistinguishable from one another. See Table 3 for additional details.
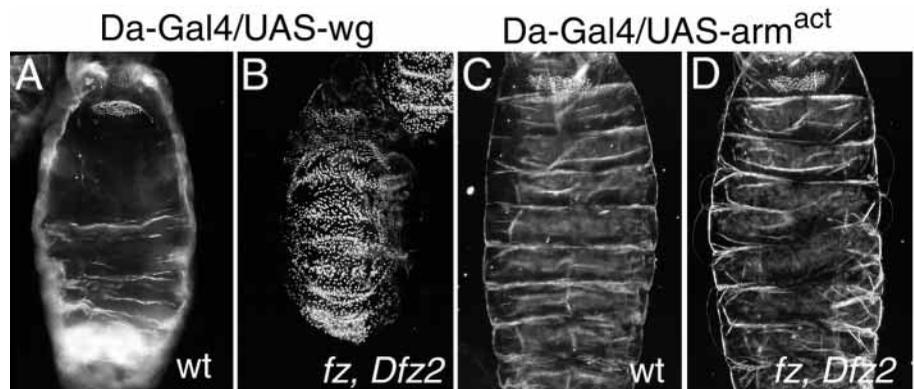
found when the *fz^P21* or *fz^D21* alleles were recombined with *Df(3L)Dfz2* but partial phenotypes were often observed in the presence of the *fz^R52* allele. The retention of partial function inferred for the *fz^R52* allele is consistent with several studies showing that, in some cases, the intracellular C terminus of Fz receptors is dispensable for Wnt signaling (Sawa et al., 1996). In two earlier genetic studies, the partial function of the *fz^R52* allele was probably responsible for the incomplete *wg*-like phenotypes that were interpreted to imply the existence of additional Wg receptors (Bhat, 1998; Muller et al., 1999).

The lack of an effect on epidermal patterning of ectopic Wg in a *fz, Dfz2* double mutant argues against either the existence of additional Wg receptors in the embryo or of a model in which Fz and Dfz2 act simply to present Wg to its bona fide receptor. In both cases, high levels of Wg might be expected to partially or completely bypass loss of Fz and Dfz2.

With respect to the apparent redundancy of Fz and Dfz2 function inferred during normal embryogenesis, we note that the experiments presented here do not rule out the possibility that in a *Dfz2* mutant Fz function or *fz* expression is altered to compensate for the absence of Dfz2, and that in a *fz* mutant Dfz2 function or *Dfz2* expression is altered to compensate for the absence of Fz.

In the embryonic epidermis, where Wg signaling has been most thoroughly studied, Wg controls the production of naked cuticle and denticle diversity. These two functions can be independently altered by different *wg* alleles, leading to the hypotheses that different cuticle fates arise either from the activation of different receptors or from different levels or spatial distributions of Wg (Hays et al., 1997). The normal or nearly normal cuticular pattern seen in *fz* or *Dfz2* single mutants and the *wg*-like cuticular phenotype seen in *fz, Dfz2* double mutants support the latter hypothesis.

Despite the near identity of *fz* and *Dfz2* action in the early embryo, it is possible that there are subtle differences between them that bear upon the different readouts of Wg activity. For example, Fz and Dfz2 might have different affinities for Wg, they might interact differentially with cofactors or proteoglycans that affect Wg signaling, or they might be used differentially in various tissues. If various developmental events were sensitive to different levels of Wg signaling, then small differences in receptor-ligand affinity or receptor density might be relevant in some tissues and not in others. For

example, in the CNS, the residual quantities of functional or partially functional protein produced from the *fz^R52* allele are sufficient for specification of some RP2 neurons, whereas it is not sufficient for specifying Eve-expressing heart precursors. Conversely, in 469-2 homozygotes, there is occasional loss of RP-2 neurons but no effect on *eve*-expressing heart precursors. It will be interesting to extend these observations in the future by studying the effects of point mutations in *Dfz2*.

Combinatorial interactions between Frizzled and Wnt family members

Earlier misexpression studies have indicated that in imaginal discs Wg can signal through Dfz2 (Zhang and Carthew, 1998; Cadigan et al., 1998) but not through Fz (Zhang and Carthew, 1998; Axelrod et al., 1998; K. M. C. et al., unpublished results). The mechanism by which the binding specificity, availability or transduction capacity of Fz is altered between embryo and imaginal disc represents an interesting question for future investigation.

The question of whether endogenous *Dfz2* is required for Wg signaling in imaginal discs cannot be addressed with the *Dfz2* deficiency described in this report, since we have not been able to obtain somatic homozygous clones in discs (H. Lin and K. M. C., unpublished results). Once more surgical mutations in the *Dfz2* gene are obtained, it will be interesting to determine whether *Dfz2* acts alone or in tandem with other Frizzled family members in these tissues.

In summary, the experiments reported here indicate that in vivo there are overlapping interactions between Frizzled and Wnt family members such that a single Wnt can interact with more than one Frizzled receptor. We infer that the converse is also true based on the available data regarding Fz function in the embryo and imaginal disc. Presumably, the promiscuous binding observed between various Wnt and Frizzled family members in cell culture experiments represents the in vitro correlate of the broad in vivo specificities inferred from genetic experiments. By extension, we predict that the 16 Wnt and 9 Frizzled proteins identified thus far in mammals are likely to exhibit complex patterns of pairwise interactions in producing the full repertoire of Wnt signaling events during mammalian development.

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