Cell, Vol. 95, 1017–1026, December 23, 1998, Copyright ©1998 by Cell Press

Use of dsRNA-Mediated Genetic Interference to Demonstrate that *frizzled* and *frizzled 2* Act in the Wingless Pathway

Jason R. Kennerdell and Richard W. Carthew*
Department of Biological Sciences
University of Pittsburgh
Pittsburgh, Pennsylvania 15260

Summary

We investigated the potential of double-stranded RNA to interfere with the function of genes in *Drosophila*. Injection of dsRNA into embryos resulted in potent and specific interference of several genes that were tested. In contrast, single-stranded RNA weakly interfered with gene activity. The method was used to determine the reception mechanism of the morphogen Wingless. Interference of the *frizzled* and *Drosophila frizzled 2* genes together produced defects in embryonic patterning that mimic loss of *wingless* function. Interference of either gene alone had no effect on patterning. Epistasis analysis indicates that *frizzled* and *Drosophila frizzled 2* act downstream of *wingless* and upstream of *zeste-white3* in the Wingless pathway. Our results demonstrate that dsRNA interference can be used to analyze many aspects of gene function.

Introduction

The Wnt family of secreted diffusible glycoproteins plays an important role in animal development (reviewed by Cadigan and Nusse, 1997). The *Drosophila* Wnt-1 ortholog Wingless (Wg) functions as an inductive signal during embryonic and imaginal development of the fruit fly. Wg is required for the determination of specific cell fates and polarity within each segmental unit of the embryo. Wg is also required for the patterning of the adult eyes, legs, and wings (reviewed by Klingensmith and Nusse, 1994).

A number of genes have been identified that have mutant phenotypes consistent with defects in Wg signaling. *porcupine* (*porc*), *dishevelled* (*dsh*), *armadillo* (*arm*), and *pangolin* (*pan*) mutant embryos have segment polarity defects similar to *wg* mutants. *zeste-white3* (*zw3*) mutants have a defect opposite that of *wg* mutants. These genes have been ordered in a genetic pathway (Noordermeer et al., 1994; Siegfried at al., 1994; Brunner et al., 1997; Van de Wetering et al., 1997). Biochemical analysis of the relationship between the protein products has strengthened this genetic picture of Wg signal transduction (Cadigan and Nusse, 1997). It is thought that Wg protein is secreted with the assistance of Porc. When cells receive a Wg signal, the cytoplasmic Dsh protein inhibits the activity of the ZW3 protein kinase. In the absence of a Wg signal, nonrepressed ZW3 produces rapid turnover of the β-catenin family member Arm. When ZW3 is repressed by Wg and Dsh, Arm protein is stabilized, and together with Pan, Arm can activate transcription of target genes.

The identification of the Wg receptor has proved to be more difficult. *Drosophila* frizzled 2 (DFz2), a member of the Frizzled family of seven-transmembrane proteins, has been shown to have activities in cultured cells that are consistent with a receptor-like function (Bhanot et al., 1996). Although no mutant in the *DFz2* gene has yet been identified, a series of experiments overexpressing full-length and truncated DFz2 support a model where DFz2 is required for Wg signaling at the dorsal/ventral boundary of the wing imaginal disc (Cadigan et al., 1998; Zhang and Carthew, 1998).

Wg has also been found to interact with the Frizzled (Fz) protein in cultured cells (Bhanot et al., 1996). However, various loss- and gain-of-function analyses of Fz have indicated that it plays no detectable role in Wg signaling in imaginal discs. Instead, Fz is required to correctly orient adult hairs, bristles, and eye facets along particular body axes (Gubb and Garcia-Bellido, 1982; Krasnow and Adler, 1994; Zheng et al., 1995; Zhang and Carthew, 1998). Wg appears not to function directly in controlling polarity of these structures (Baker, 1988a; Struhl and Basler, 1993; Reifegerste et al., 1997; Heberlein et al., 1998). These data support a specificity of DFz2 for Wg signaling in imaginal disc development.

During embryogenesis, Wg has multiple functions in pattern formation. One signaling role for Wg is to maintain the expression of the *engrailed* gene in epidermal cells immediately posterior to those cells expressing Wg (DiNardo et al., 1988; Martinas Arias et al., 1988). The maintenance of *engrailed* expression by Wg occurs at an early phase of embryogenesis (Bejsovec et al., 1991; Heemskerk et al., 1991). Later, Wg specifies the differentiation of naked cuticle and is required for generating the diversity and polarity of denticles in each segment (Baker, 1988b; Bejsovec et al., 1991; Bejsovec and Wieschaus, 1993; Lawrence et al., 1996).

To address the mechanism of Wg signal transduction, we have introduced double-stranded RNA (dsRNA) into embryos to interfere with gene function. Recently, dsRNA was found to be a potent and specific inhibitor of gene activity in the nematode *Caenorhabditis elegans* (Fire et al., 1998). Therefore, dsRNA might be a useful method to determine gene function in other organisms. We introduced into *Drosophila* embryos dsRNA corresponding to four genes with previously defined functions. We show that dsRNA potently and specifically inhibits the activities of *wg*, *fushi tarazu* (*ftz*), *even-skipped* (*eve*), and *tramtrack* (*ttk*). In contrast, single-strand RNA had at most a weak effect. Levels of dsRNA stoichiometric with endogenous transcripts were sufficient to produce interference. These results suggest a striking conservation in the mechanism of dsRNA-mediated genetic interference between nematodes and insects.

We used dsRNA corresponding to *frizzled* (*fz*) and *Dfz2* to determine whether they transduce the Wg signal in the embryonic epidermis. Interference of both genes results in a *wg*-like phenotype. We injected dsRNA into

*To whom correspondence should be addressed (e-mail: carthew@pop.pitt.edu).

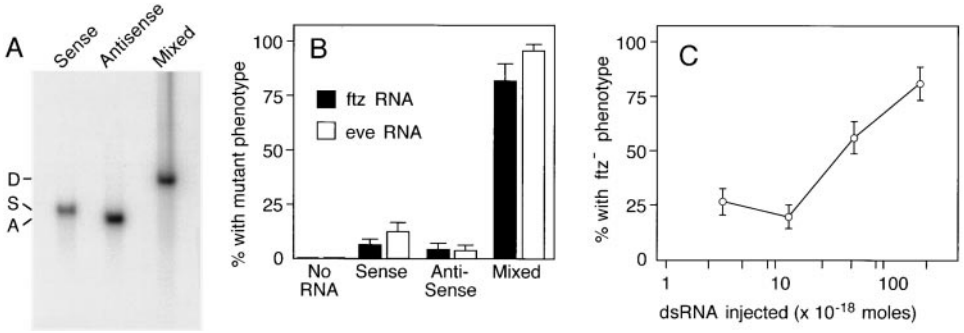

Figure 1. Effects of Sense, Antisense, and Mixed RNAs on *ftz* and *eve* Activities

(A) ^{32}P-labeled antisense and sense *ftz* RNAs were resolved by electrophoresis in a native 1% agarose gel. Aliquots of antisense and sense RNAs were run separately or the two RNAs were mixed and annealed before loading on the gel. A, antisense monomer; S, sense monomer; D, duplex RNA formed from the sense and antisense RNAs.

(B) Wild-type embryos were injected either with buffer alone, sense *ftz* RNA, sense *eve* RNA, antisense *ftz* RNA, antisense *eve* RNA, mixed *ftz* RNA, or mixed *eve* RNA. All embryos received 0.2 fmol of RNA. Relative interference activity is presented as the percentage of injected survivors that exhibit a *ftz* or *eve* mutant phenotype. Error bars represent standard deviations.

(C) Dose dependence of RNA-mediated interference of *ftz*. Different concentrations of mixed *ftz* RNA were injected at a constant volume into wild-type embryos. The percentage of injected survivors that exhibit a *ftz* phenotype were quantified. Error bars represent standard deviations.

mutant embryos as a means to test for genetic epistasis. We show that *fz* and *Dfz2* act upstream of *zw3* and downstream of *wg* to affect cuticle differentiation. Thus, dsRNA interference may be a valuable system with which to understand aspects of gene function in many organisms.

Results

RNA-Mediated Interference in *Drosophila*

In these studies, we have adapted dsRNA-mediated genetic interference for use in *Drosophila*. dsRNA-mediated genetic interference is based on the observation that dsRNA injected into adult *C. elegans* nematodes specifically blocks gene activity (Fire et al., 1998). The effects of interference were observed in the injected worms and their progeny. Moreover, only a few dsRNA molecules were required in each affected cell, indicating that its potency was due to some amplifiable process. To determine whether dsRNA-mediated interference can occur in *Drosophila*, we synthesized RNA in vitro, allowed it to anneal, and injected it into syncytial blastoderm embryos. The *ftz* and *eve* genes were chosen for initial characterization of the method based on several criteria. Both genes are required for embryonic segmentation (Scott et al., 1983; MacDonald et al., 1986). Transcription of *ftz* and *eve* begins approximately 90 to 120 min after egg laying, which corresponds to a time 10 to 60 min after dsRNA injection (Hafen et al., 1984; MacDonald et al., 1986). Although both genes function in the first few hours of embryogenesis, null mutant animals survive to the end of embryogenesis and exhibit segmentation defects in their cuticle. Finally, mutants with reduced activity of either *ftz* or *eve* produce increasingly severe phenotypes (Jurgens et al., 1984; Nusslein-Volhard et al., 1984), such that a semiquantitative relationship exists between genotype and phenotype.

Antisense and sense RNAs for each gene were synthesized and annealed. Electrophoretic analysis indicated that the material was predominantly double stranded, since its mobility was shifted to a position expected for dsRNA of that size (Figure 1A). This RNA undergoes a temperature-dependent hyperchromicity of 42% (A_{280}) with a transitional midpoint at 86°C (data not shown). Injection of either *ftz*- or *eve*-annealed RNA into wild-type embryos effectively interfered with gene activity as demonstrated by cuticle phenotypes characteristic of *ftz* or *eve* mutants (Figures 1B, 2D, and 2E). In contrast, antisense or sense RNAs injected separately had an order-of-magnitude weaker interference activity than annealed RNA (Figure 1B). Animals injected with buffer alone had no detectable interference. When a decreasing amount of *ftz*-annealed RNA was injected, interference activity declined also, though interference was still detectable at the lowest dose (Figure 1C). The abundance of each RNA strand at this dose was calculated to be about 2 million molecules per injected embryo. Assuming uniform distribution of RNA, the original injected material would be diluted to about 30 molecules per cell. Thus, dsRNA is a robust inhibitor of gene activity in *Drosophila*, comparable in its potency to that observed in *C. elegans*.

The phenotypes produced by ds-*ftz* and ds-*eve* RNAs were highly specific. Injected animals exhibited cuticle defects indistinguishable from *ftz* and *eve* loss-of-function mutants (Figures 2C–2E). The phenotypes varied significantly between individuals, possibly due to variability in the injected dose. At high doses of ds-*ftz* RNA, we observed the majority of animals (16 of 22) exhibiting the null mutant phenotype (Figure 2C). At lower doses of ds-*ftz* RNA, the majority of animals (13 of 14) exhibited localized or patchy interference (Figure 2F). This localized phenotype was consistent with loss of *ftz* activity. Even within a group of animals given the same dose, variation in phenotype was apparent. Some ds-*eve* RNA–treated animals (7 of 44) exhibited a lawn of denticles characteristic of the known null mutant (data not shown), while the remaining animals exhibited a complete pair-rule phenotype (27 of 44) or localized pair-rule phenotype (10 of 44) characteristic of partial loss of *eve* function

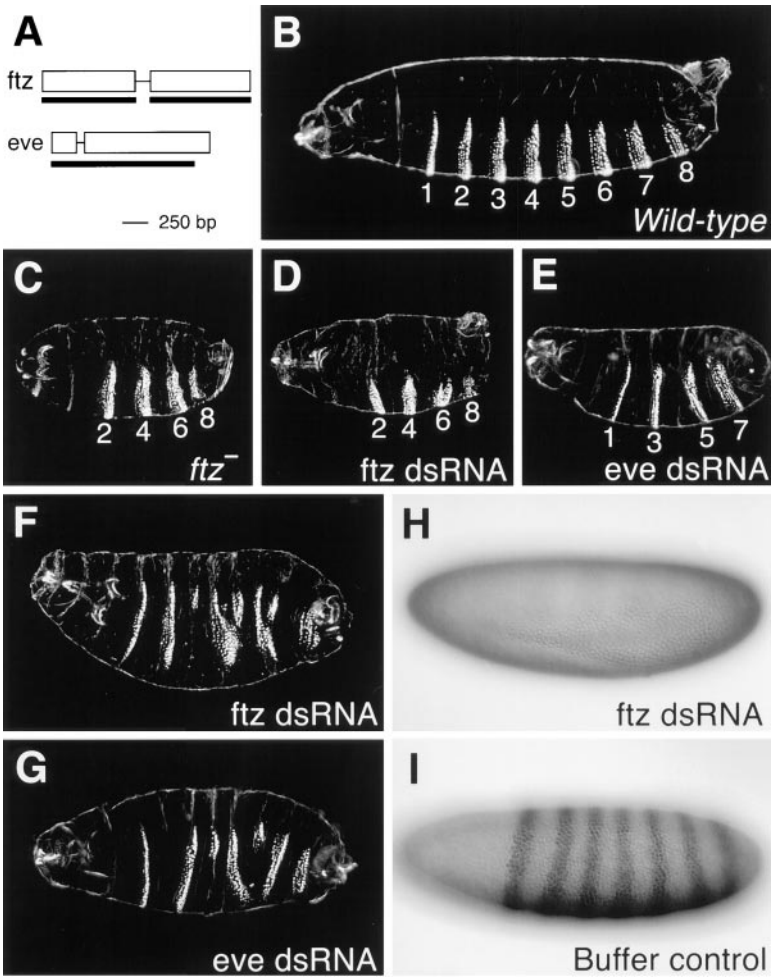

Figure 2. RNA Interference of *ftz* and *eve* Activities in Embryos

(A) Structure of the *ftz* and *eve* genes and their interfering RNA segments. Open boxes, exons; lines, introns. Interfering RNAs are indicated by filled bars below each gene.

(B–G) Lateral or ventral views of cuticle preparations. (B) In the wild-type embryo, eight abdominal segments (numbered 1–8) secrete cuticle containing clustered rows of denticles that appear white under dark field. (C) ftz^{13} homozygous mutant embryos are missing the odd-numbered abdominal segments. (D) A wild-type embryo injected with ds-*ftz* RNA has a phenotype similar to that of ftz^{13}. (E) A wild-type embryo injected with ds-*eve* RNA is missing the even-numbered abdominal segments. (F and G) ds-*ftz* and ds-*eve* RNAs can produce localized loss of segments that highlight their specificities. The cuticle in (F) is missing most of segments A3, A5, and A7, while the cuticle in (G) is missing all of A2 and most of A4 and A6.

(H and I) Ftz immunostaining in stage 5 wild-type embryos injected with ds-*ftz* RNA (H) or buffer only (I). Ftz expression has disappeared from the RNA-injected embryo but is detected in a normal seven-stripe pattern in the buffer-injected embryo.

(Figures 2E–2G). Since both *ftz* and *eve* are expressed in cells spanning 60% the embryo's length, the complete phenotypes observed indicate that interference can occur in cells throughout the embryo.

The observed interference is at the level of gene expression. Little or no endogenous Ftz protein was observed in embryos injected with ds-*ftz* RNA (Figure 2H). In contrast, embryos injected with buffer exhibited a normal pattern of Ftz protein expression (Figure 2I).

We further assessed target specificity of dsRNA interference using the *tramtrack* (*ttk*) gene. *ttk* encodes two alternatively spliced proteins with different pairs of zinc fingers (Read and Manley, 1992). Both proteins are together required for development of chordotonal (ch) organs of the embryonic nervous system. As shown by Guo et al. (1995), the number of ch neurons is greater in *ttk* mutant embryos. Compared with wild type, the number of neurons in each lateral cluster of five ch organs, termed lch5, is approximately doubled at the expense of other organ cell types (Figures 3A–3D). *ttk* is expressed in ch cells when the sensory organ precursors are determined at approximately stage 11–12 (Jarman et al., 1993; Guo et al., 1995). Since this corresponds to a time approximately 6 to 7 hr after we normally injected dsRNA into embryos, interference of *ttk* would also allow us to test whether dsRNA can persist for several hours. Persistence is an important issue because many endogenous RNAs are rapidly degraded in fly embryos. We prepared dsRNA corresponding to exons common in both *ttk* transcripts, injected embryos with the RNA, and examined the lch5 organ in each abdominal segment. All injected embryos (37 of 37) exhibited lch5 organs with increased numbers of neurons (Figures 3E and 3F). The potency of this effect was profound, with 90% (33 of 37) having all of their lch5 organs affected. Besides the defects in the peripheral nervous system, we also observed that almost all embryos failed to undergo dorsal closure and head involution, two defects observed in *ttk* mutants (Guo et al., 1995; compare Figures 3A and 3E). A significant number of injected animals (26 of 37) exhibited defects more profound than the previously characterized *ttk* null mutants (Figures 3G and 3H). These defects included extensive hyperplasia of the nervous system reminiscent of mutants in the "neurogenic" class of genes that include *Notch*. The dsRNA covers the highly conserved BTB/POZ domain and may have interfered with other BTB/POZ domain genes. Alternatively, the dsRNA may have interfered with both maternally supplied *ttk* transcripts and zygotic *ttk* activity. Since the described *ttk* mutants have only removed zygotic activity, it is possible that removal of both maternal and zygotic *ttk* activities would produce

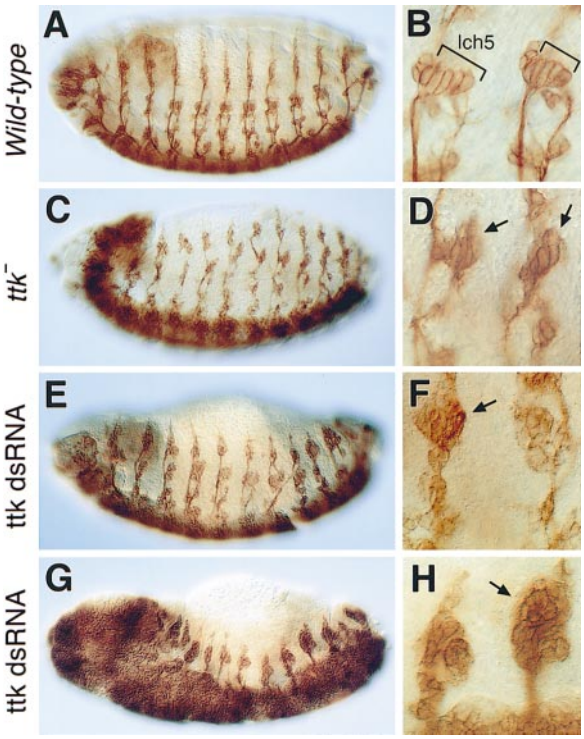

Figure 3. RNA Interference of *ttk* Gene Function

(A, C, E, G) Lateral view of stage 15 embryos immunostained with anti-Neuroglian that stains neurons. (B, D, F, H) High magnification view of same embryos showing midlateral regions of segments A5 and A6. (A and B) Wild-type embryo injected with buffer. Brackets highlight the five neural cells in each lch5 chordotonal organ. (C and D) ttk^{1e11} homozygous mutant embryo. The number of lch5 neurons (arrows) is approximately doubled. (E–H) Wild-type embryos injected with ds-*ttk* RNA. Extra neurons are formed in the lch5 organs (arrows) of some injected embryos (F). Other embryos exhibit a neurogenic phenotype (G) with a highly condensed PNS containing many extra neurons (H).

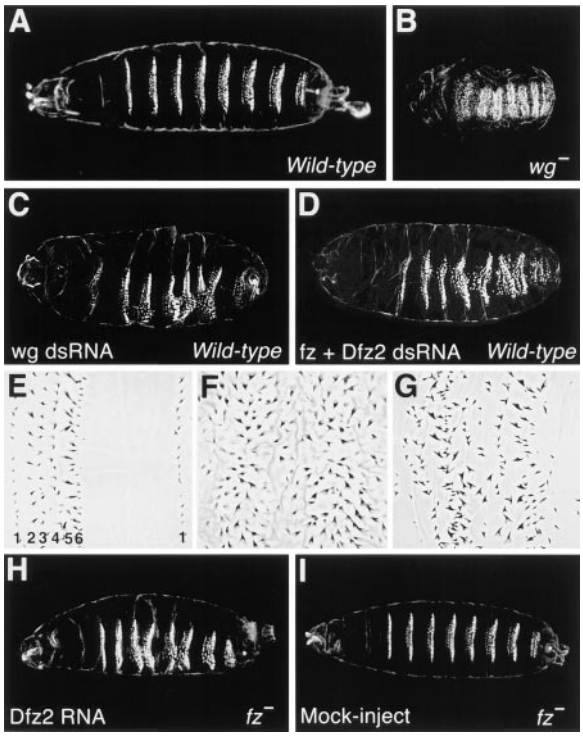

Figure 4. Interference of *Dfz2* and *fz* Activities Produces a Segment-Polarity Phenotype

Ventral view of cuticle preparations.

(A) Mock-injected wild-type embryo. Anterior cells of each segment secrete cuticle with denticles, and posterior cells secrete naked cuticle.

(B) wg^{1-8} homozygous mutant embryo. Posterior cells are transformed to anterior fates and secrete denticles.

(C) Wild-type embryo injected with 0.4 fmol of ds-*wg* RNA. Localized areas of cuticle exhibit a phenotype similar to that of wg^{1-8}.

(D) Wild-type embryo injected with 0.2 fmol each of ds-*fzA* and ds-*Dfz2A* RNAs. The phenotype is very similar to that seen in (C).

(E) High magnification view of mock-injected wild-type embryo showing one abdominal segment. Note the six rows of denticles in each segment, numbered 1–6. Denticles in each row are uniformly the same size and oriented in the same direction. The anterior region of each segment constitutes denticle rows 2–6, and the posterior region constitutes naked cuticle plus denticle row 1.

(F) High magnification view of wg^{1-8} mutant embryo. Most of the denticles resemble the large type found in a normal row 5. Denticle orientation is often reversed or aligned towards the ventral midline.

(G) Wild-type embryo injected with both ds-*fzL* and ds-*Dfz2L* RNAs. Denticle transformations and orientation defects resemble those seen in wg^{1-8}.

(H and I) fz^1 homozygous mutant embryos injected with 0.2 fmol ds-*Dfz2L* RNA (H) or mock injected (I). fz^1 males and females were crossed to produce the mutant embryos. Thus, 100% of the embryos had a mutant maternal contribution as well as a mutant zygotic genotype. Mock-injected fz^1 embryos looked normal with one exception. A single embryo (1 of 100) exhibited supernumerary denticles in a small part of one segment. *Dfz2L* RNA–injected fz^1 embryos exhibited segment-polarity phenotypes 6-fold more frequently than *Dfz2L* RNA–injected wild-type embryos (6 of 35 embryos vs. 3 of 141 embryos).

a neurogenic phenotype. Consistent with this possibility, *ttk* mutant cuticle phenotypes resemble *Notch* mutants (Xiong and Montell, 1993).

Fz and DFz2 Function Redundantly in Embryonic Patterning

The potency and specificity of dsRNA interference on gene activity suggested that it might be a useful means to eliminate *Dfz2* activity. Although the null phenotype of *Dfz2* is unknown, we reasoned that if *Dfz2* encodes the Wg receptor, then its mutant phenotype should resemble *wg* loss-of-function mutants. Larvae that lack *wg* activity are completely covered with denticles on the ventral cuticle, unlike wild-type larvae in which ventral cuticle is an alternating pattern of naked cuticle and denticles (Figures 4A and 4B). When dsRNA corresponding to the *wg* gene was injected, the region around the site of injection exhibited a *wg*-like mutant phenotype, and the remainder of the embryo was wild type (Figure 4C). Surprisingly, no animals exhibited a null phenotype despite the injection of twice as much dsRNA as for other genes. The RNAi effect was localized, and the range of phenotypes was limited by the size of the region with ectopic denticles. When dsRNA corresponding to the 5′ UTR of *Dfz2* was injected, no effect on denticle patterning was observed (Figure 5). We also did not observe ectopic denticles in embryos injected with dsRNA corresponding to the 5′ UTR of the *fz* gene. In contrast, an equimolar mixture of ds-*fz* and ds-*Dfz2* RNAs caused localized transformation of naked cuticle

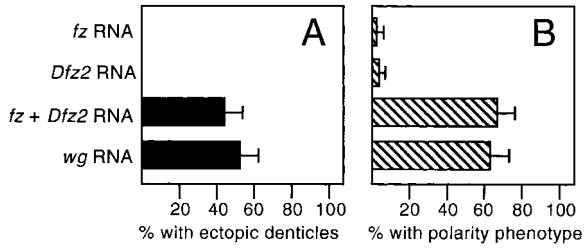

Figure 5. Synergistic Effect of *fz* and *Dfz2* Interference

Wild-type embryos were injected with dsRNA: either 0.4 fmol ds-*fzA* RNA, 0.4 fmol ds-*Dfz2A* RNA, 0.2 fmol each of ds-*fzA* and ds-*Dfz2A* RNAs, or 0.4 fmol ds-*wg* RNA. Relative interference activity is presented in (A) as the percentage of injected survivors that have ectopic ventral denticles and in (B) as the percentage of injected survivors that have defects in denticle polarity. Embryos were scored positive for ectopic denticles if naked cuticle in more than 1/4 of a single segment was transformed. They were scored positive for polarity defects if denticles in one or more abdominal segments were oriented incorrectly. The low frequency (2%–4%) of polarity defects seen with ds-*fzA* and -*Dfz2A* RNAs alone may be due to the injection process, since injection of buffer gave a similar level of polarity defects. Error bars represent standard deviations.

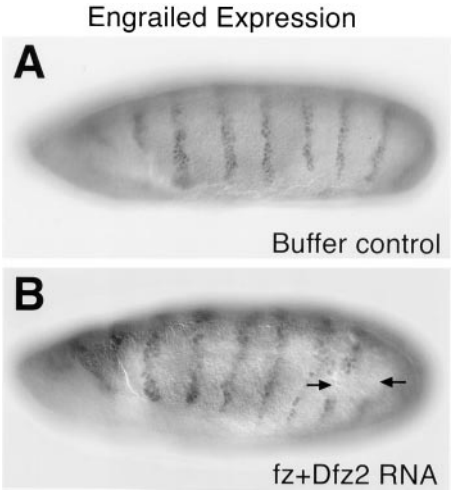

Figure 6. Interference of *fz* and *Dfz2* Blocks *engrailed* Expression

(A) Wild-type *engrailed* is expressed in the posterior compartment of each segment. Shown is a dorsolateral view of a stage 10 embryo that had been injected with buffer.

(B) A stage 10 embryo that had been injected with 0.1 fmol each of ds-*fzL* and ds-*Dfz2L* RNAs. Cells in the lateral (arrows) region have lost *engrailed* expression. In other injected embryos, midventral and dorsal cells also lost *engrailed* expression (not shown).

into denticles (Figures 4D and 5). The RNAi effect was limited to the site of injection even at high doses of dsRNA, and its potency was highly similar to the potency of ds-*wg* RNA. Denticles in the affected regions resembled those typical of the fifth row in a wild-type abdominal segment, and the denticles were oriented either toward the midline or along the anteroposterior axis with reversed polarity (Figures 4E–4G and 5). These features are precisely those observed in *wg* mutant embryos and embryos treated with ds-*wg* RNA.

Engrailed expression initiates normally in *wg* mutants but fails to be maintained (DiNardo et al., 1988; Martinas Arias et al., 1988). To examine whether *fz* and *Dfz2* have a similar function, we injected embryos with ds-*fz* and ds-*Dfz2* RNAs. After further development, the embryos were stained with an anti-Engrailed antibody. Expression of *engrailed* was absent in lateral ectoderm within the affected region (Figure 6). In embryos that were injected with buffer, *engrailed* expression in lateral ectoderm was easily detected. This discontinuous loss of *engrailed* expression resembled loss of functional *wg* (Bejsovec and Martinas Arias, 1991).

The interfering activity of ds-*fz* and ds-*Dfz2* RNA mixtures could mean that the *fz* and *Dfz2* genes act redundantly, and the activities of both genes must be blocked before a phenotype is observed. Alternatively, it could reflect some other synergy between the injected RNAs. ds-*Dfz2* RNA was injected alone into embryos mutant for *fz* and was found to possess interfering activity that was comparable to the interfering activity of mixed ds-*fz* and ds-*Dfz2* RNAs (Figure 4H). In contrast, mock-injected embryos mutant for *fz* had a negligible segment-polarity phenotype (Figure 4I). These data are most consistent with the *fz* and *Dfz2* genes acting redundantly to pattern the ventral epidermis.

Fz and DFz2 Act Downstream of Wg but Upstream of ZW3

Experiments in cell culture had suggested that DFz2 acts as a receptor in the Wg signal transduction pathway (Bhanot et al., 1996). We wanted to know if Fz and DFz2 act between Wg and the intracellular components of its signal transduction pathway. Genetic epistasis can determine the order of action of genes in a common pathway. If *fz* and *Dfz2* function downstream of *wg*, then interference of *fz* and *Dfz2* activities should suppress activating mutations of *wg*. A transgenic strain that expresses high levels of Wg in all epidermal cells causes those cells to secrete naked cuticle (Lawrence et al., 1996; Figure 7A). We used this strain to determine whether *fz* and *Dfz2* are required for *wg* action. When these animals were injected with ds-*fz* and ds-*Dfz2* RNAs, the formation of ectopic naked cuticle was suppressed (Figure 7B; Table 1). The injected transgenic embryos were distinct from wild-type embryos injected with RNA (Figure 4D) and from the uninjected transgenic strain (Figure 7A). They resembled wild-type embryos injected with dsRNA in that they had denticles alternating with naked cuticle plus some localized patches of continuous denticle lawn. However, they did not usually have the complete complement of denticles (Table 1). We attribute this incomplete suppression to the fact that interference of *fz* and *Dfz2* is primarily localized to regions close to the site of injection. Nevertheless, this result provides genetic evidence for a function of *fz* and *Dfz2* downstream of *wg*.

Transduction of a Wg signal antagonizes the ZW3 kinase, which functions to modulate levels of Arm. Do *fz* and *Dfz2* act between *wg* and *zw3*, as would be predicted for the Wg receptor? Loss of *zw3* activity results in all epidermal cells adopting posterior segmental fates, and mutant embryos lack ventral denticles (Figure 7C). When *zw3* mutant embryos were injected with ds-*fz* and ds-*Dfz2* RNAs, there was no change in their phenotype; they resembled *zw3* embryos (Figure 7D; Table 1). The similarity of the phenotypes of *zw3* with or without *fz* and *Dfz2* interference suggests that *fz* and *Dfz2* function upstream of *zw3* in *wg* signaling.

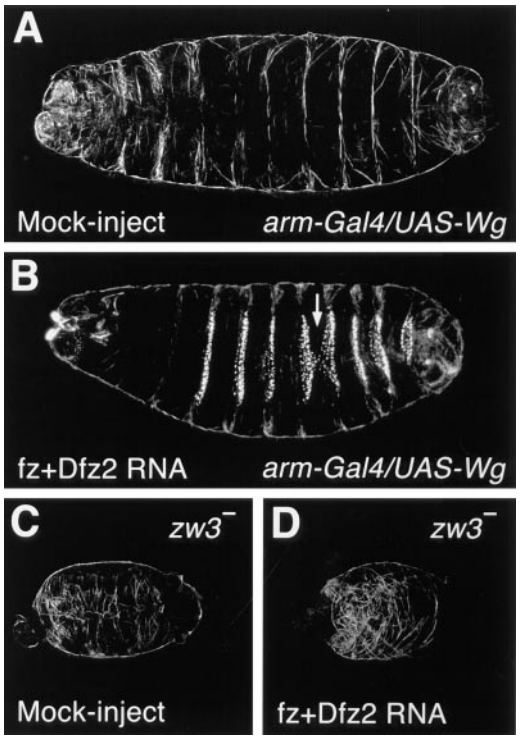

Figure 7. Effects of *fz* and *Dfz2* Interference on *wg* and *zw3* Cuticle Phenotypes

(A) A mock-injected Arm-Gal4/UAS-Wg^{ts} embryo. Although segmentation has occurred, no ventral denticles are present due to ectopic expression of Wg (see Experimental Procedures).

(B) An Arm-Gal4/UAS-Wg^{ts} embryo that had been injected with 0.1 fmol each of ds-*fzL* and ds-*Dfz2L* RNAs. This embryo shows a restored pattern of denticles similar to wild-type plus ectopic denticles in regions where naked cuticle should be secreted (arrow).

(C) A mock-injected *zw3*$^{M11\text{-}1}$ embryo. No ventral denticles are present.

(D) A *zw3*$^{M11\text{-}1}$ embryo that had been injected with 0.1 fmol each of ds-*fzL* and ds-*Dfz2L* RNAs (see Experimental Procedures).

Discussion

dsRNA Interference in *Drosophila*

We have found that dsRNA is a potent and specific inhibitor of gene activity in *Drosophila*. To date, we have attempted to interfere with seven genes (this study and unpublished data) and successfully generated loss-of-function phenotypes for all. Thus, RNAi works efficiently in *Drosophila*. Several features of RNA interference are common to both *Drosophila* and *C. elegans*. First, dsRNA is much more effective at producing interference than either individual single strand. Second, the presence of a few dsRNA molecules per cell is sufficient to interfere with genes producing much greater levels of mRNA. We have observed interference of *ftz* activity with as little as 30 molecules per cell. A rough estimate of *ftz* mRNA abundance is 150 molecules per cell, or an order of magnitude more than the inhibitory dsRNA. Thus, dsRNA at substoichiometric levels is sufficient to interfere with gene activity. Third, a sequence shared between several closely related genes may interfere with several members of the gene family. We found that dsRNAs corresponding to the 5′ UTRs of *fz* and *Dfz2* had no interfering activities on their own (Figure 5), whereas dsRNAs corresponding to coding sequences shared by *fz* and *Dfz2* had weak but significant interfering activities (data not shown). Another feature of RNAi in *C. elegans* is that dsRNA is taken up by most or all cells, including germ cells that pass on the interference to progeny worms. We injected dsRNA into syncytial blastoderms that have no cell membranes delimiting the early zygotic nuclei. Therefore, we do not know if dsRNA can also be taken up by *Drosophila* cells. Moreover, our limited study of germline interference has not yielded any observable transmission of interference through the germ line (data not shown). However, the genes that we initially chose for interference produce lethal loss-of-function phenotypes that may also select against viable germline transmission. Use of dsRNA against benign transgenes (e.g., *lacZ*) may provide more information about its persistence.

We observed variability in the interference activities of different dsRNAs. While some generated null phenotypes with high efficiency (*ttk, ftz, eve*), others only generated embryos with mosaic patterns of wild-type and affected tissues (*wg, fz, Dfz2*). The mosaicism of the RNAi phenotype and its localization close to the site of injection suggest that there is loss of gene product in some cells and not others. Several factors may play a role in this variability. It is possible that a low level of gene expression escapes interference, and some phenotypes (such as segment polarity) are less sensitive to the level of gene activity. Alternatively, differences in mRNA localization may affect interference. All of the genes in our mosaic class encode secreted or membrane proteins with mRNAs predicted to associate with the rough ER. Finally, abundant mRNAs may be more sensitive to interference than mRNAs of lower abundance.

The mechanism for dsRNA interference is unknown.

Table 1. Epistasis Analysis of *fz* and *Dfz2*

Genotype	Treatment	Mean Number of Abdominal Denticle Rows per Animal	Number of Animals Scored
Arm-Gal4/UAS-Wg^{ts}	Mock injected	5.0	101
	ds-*Dfz2L* + ds-*fzL*	17.6	37
zw3$^{M11\text{-}1}$	Mock injected	0.5	24
	ds-*Dfz2L* + ds-*fzL*	0.5	63

Embryos were scored for the number of denticle rows in each abdominal segment. These numbers were totaled and averaged for the number of embryos scored. A normal embryo has 46 rows of abdominal denticle rows.

If the mechanism is the same for both flies and nematodes, then work from *C. elegans* points to a posttranscriptional mechanism (Montgomery and Fire, 1998). In this regard, dsRNA inhibition of *ttk* activity generated phenotypes, suggesting that both zygotic and maternal *ttk* activities were sensitive to interference. Since *ttk* mRNA transcripts are deposited maternally in eggs for use in zygotes (Read and Manley, 1992), this suggests that dsRNA may cause transcript degradation or block translation. If so, then interference is amplified by some mechanism, possibly via catalysis. dsRNAs corresponding to either the 5′ or 3′ UTR of a gene appear to be potent inhibitors. This result makes it more unlikely that translational arrest plays a role in the interference mechanism.

dsRNA Interference in Other Species?

The similar effects of dsRNA on gene activity in a nematode and insect species suggest that most or all other species sharing these lineages are also susceptible to its effects. Moreover, it supports the hypothesis that dsRNA interference controls gene expression in many animals, including vertebrates (Montgomery and Fire, 1998). Is there a physiological role for dsRNA in regulating gene expression? It might be used as a specific cellular defense mechanism against viral infection. Or it might be a general mechanism to regulate normal gene expression. Identification of *trans*-acting factors required for interference may aid in its understanding.

As a genetic method for nongenetic organisms, dsRNA may have broad potential. Even for *Drosophila melanogaster*, dsRNA has considerable utility. It is estimated that only one-third of the 12,000 fly genes can mutate to cause a detectable phenotype (Miklos and Rubin, 1996). Genetic redundancy likely accounts for the majority lacking a phenotype. However, injection of multiple dsRNAs into a single animal, as done in our study with *fz* and *Dfz2*, might be a simple means to overcome this redundancy and generate mutant phenotypes. Finally, dsRNA interference experiments on other invertebrate species could have consequences for the field of comparative evolution and development that relies upon comparing gene structure, expression, and function in related species. Although techniques for altering gene function in arthropod species are being developed (Heldens et al., 1997), dsRNA interference may become a useful application for this field.

Functions of Fz and DFz2 in Wg Signaling

This study demonstrates that Fz and DFz2 function redundantly, downstream of Wg and upstream of ZW3 in the establishment of segment polarity. We propose that Fz and DFz2 function directly in the Wg signaling pathway based on epistasis analysis. Although it is possible that Fz and DFz2 act in a parallel pathway, this seems unlikely given cell culture experiments that indicate Fz and DFz2 interact directly with Wg proteins (Bhanot et al., 1996). Are Fz and DFz2 the only receptors for Wg? The inability of ds-*fz* and ds-*Dfz2* RNAs to generate a null *wg*-like phenotype might suggest so. However, two lines of evidence make it unlikely that another receptor has a major function in parallel with Fz and DFz2. First, ds-*wg* RNA also fails to produce a null *wg* phenotype and produces a similar phenotype to ds-*fz* and ds-*Dfz2* RNAs. Thus, the partial inhibition is more likely a limitation of RNAi in blocking certain genes or genetic pathways. Second, ds-*fz* and ds-*Dfz2* RNAs that include a region shared by all members of the *frizzled* gene family inhibit embryonic patterning to the same degree as ds-*fz* and ds-*Dfz2* RNAs that correspond to the nonconserved 5′ UTR regions. These results suggest that another receptor would play a minor role, if any, in Wg signaling. Whether there is a coreceptor for the Frizzled family (like *patched* is for *smoothened*) remains to be determined.

There are similarities between how Wg signals to cells in the embryonic epidermis and wing discs. Several studies suggest that Wg acts as a morphogen in both tissues (Struhl and Basler, 1993; Lawrence et al., 1996; Zecca et al., 1996). Moreover, it utilizes the same signal transduction pathway in responding cells (Cadigan and Nusse, 1997). However, there are significant differences between how Wg works in the two tissues. In the wing disc, Wg specifies cell fate decisions but has no apparent role in controlling planar polarity of wing cells (Baker, 1988a; Zecca et al., 1996). In the embryonic epidermis, Wg specifies cell fate decisions and controls planar polarity of cells. This planar polarity is manifested by the orientation of denticles along the anteroposterior axis, which is disrupted in *wg* mutants or can be redirected by *wg* misexpression (Baker, 1988b; Bejsovec et al., 1991; Lawrence et al., 1996). There is a second major difference between wing and embryo. In the wing, DFz2 and not Fz mediates the Wg signal. Misexpression of DFz2 increases the zone of Wg responsiveness in the wing, but Fz misexpression has no effect (Cadigan et al., 1998; Zhang and Carthew, 1998). Null *fz* mutants do not perturb cell fate decisions attributable to Wg (Adler et al., 1990). In the embryo, we find both Fz and DFz2 are required to mediate the Wg signal. Inhibition of both genes is sufficient to disrupt planar polarity and epidermal cell differentiation, whereas inhibition of each gene singly has no effect. Our data is also consistent with experimental results in which Fz was overexpressed in embryos (Tomlinson et al., 1997).

How can Wg, Fz, and DFz2 generate both polarity and cell fate responses in embryos and not in wing discs? One possibility is that in embryos they directly specify cell fates and indirectly affect cell polarity. For instance, they specify the diverse pattern of denticle types that might then determine overall denticle polarity. Another possibility is that distinct domains of Wg activate different cell responses by interacting with receptors in qualitatively different ways (Hays et al., 1997). Access to some Wg domains might be limiting in some tissues and not others. A third possibility is that Wg ligand–receptor interactions are quantitatively different in various tissues. A fourth possibility is that intrinsic factors couple Wg-bound Frizzled proteins to a particular cell response, and these factors are differentially active in various tissues. We think it unlikely that Dsh is one of these factors, since Dsh can transduce both polarity and cell fate signals depending upon its differential activation (Axelrod et al., 1998). Perhaps the specificity factors signal to Dsh such that it is coupled to one pathway or

another when Wg binds to a Frizzled protein. The cell surface Notch receptor has been proposed to play an important role in controlling Wg signal transduction (Axelrod et al., 1996), possibly even acting as a Wg receptor (Couso and Martinas Arias, 1994). However, its precise function in mediating Wg signals remains unclear.

Most ligands pair with specific receptors, and each pairing remains fixed for different tissues and different developmental stages. Wg appears to be an exception to this general rule. What is the significance behind Wg's diverse signaling properties? By adding greater flexibility in the competence of cells to respond to Wg, more diverse responses to a single ligand can be generated. Competence may be modified by changing the number of potential receptors and their ability to trigger more than one transduction pathway. Another reason for this diversity might be related to the function of Wg receptors in shaping the concentration gradient of Wg in a tissue. In the wing disc, high levels of DFz2 stabilize extracellular Wg and allow it to range farther from its source than in the absence of DFz2 (Cadigan et al., 1998). Thus, if more than a single Frizzled protein can stabilize Wg, the combination of multiple receptor expression patterns might determine the Wg gradient. This simple combinatorial mechanism could potentially generate a broad range of gradient curves for a single ligand.

Experimental Procedures

RNA Synthesis

In most cases, the templates used for RNA synthesis were linearized plasmids. The templates used for synthesis of ds-*wg*, ds-*fzA*, ds-*Dfz2A*, and ds-*ttk* RNAs were products of PCR reactions using primers designed to amplify limited regions of cDNA sequence for each gene. Each primer contained a T7 promoter sequence on its 5′ end (TAATACGACTCACTATAGGGAGACCAC) such that sense and antisense RNAs could be synthesized simultaneously from a single PCR-derived template. All templates were purified using either GENECLEAN (Bio 101) or phenol/chloroform extraction and ethanol precipitation.

RNAs were synthesized using either T7, T3, or SP6 RNA polymerase (Ausubel et al., 1993). For most RNAs, the synthesized products corresponded to sequence from contiguous exons. RNAs were complementary to contiguous cDNA sequence spanning from nucleotides: 901–2837 for ds-*ftz* (GenBank accession X00854); 357–1214 for ds-*ttk* (GenBank accession X71626); 2405–2581 for ds-*wg* (GenBank accession M17230); 748–1088 for ds-*fzA* and 374–2360 for ds-*fzL* (GenBank accession X54646); 800 nucleotides of 5′ UTR for ds-*Dfz2A* (Zhang and Carthew, 1998); and 800 nucleotides of 5′ UTR plus coding sequence 1–1030 for ds-*Dfz2L* (GenBank accession U65589). ds-*eve* RNA was complementary to genomic DNA 111–1464 (GenBank accession M14767), which includes a small intron.

DNase treatment of *ftz* RNA preparations had no effect on the interference activity of *ftz* RNA (data not shown) and was not performed on some of the RNA preparations. It was unecessary to perform an annealing reaction on RNA products that were derived from the double T7-PCR templates, since these RNAs self-annealed during synthesis (data not shown). All other RNA products were extracted with phenol/chloroform, ethanol precipitated, and dissolved in annealing buffer (1 mM Tris [pH 7.5], 1 mM EDTA). To anneal, equimolar quantities of sense and antisense RNAs were mixed in annealing buffer to a final concentration of 0.45 μM each. Small aliquots (11.1 μL) of the mixture were heated in a 150 mL beaker of boiling water for 1 min, at which time the beaker was removed from the heat source and allowed to cool to room temperature for 18 hr. All RNA aliquots were then stored as an ethanol precipitate at −80°C until immediately before use. RNA precipitates were dissolved in injection buffer (Rubin and Spradling, 1982) to a final concentration of no greater than 5 μM.

Some of the RNA preparations were tested by native agarose gel electrophoresis in TBE, in which case 6–10 μg of RNA was electrophoresed and stained with ethidium bromide. Alternatively, RNA was trace labeled with ^{32}P-ATP during synthesis, and electrophoresed products were visualized by autoradiography. Only preparations in which the electrophoretic mobility of most of the RNA was shifted to that expected for dsRNA of the appropriate length were used.

Injections

Needles were baked to remove RNase. Embryos were collected over a 60 min period at 25°C, dechorionated, and attached to a coverslip coated with either rubber cement (when embryos were later analyzed by cuticle morphology) or a heptane extract of double-stick tape (when embryos were later analyzed by histochemistry). Embryos were then dessicated and covered in 700 halocarbon oil. Embryos were injected at the syncytial blastoderm stage. Injection location was on the ventral side in the posterior domain extending from 50%–75% egg length. The RNA solution was injected by a pneumatic picopump (World Precision Instruments). The average injection volume was 85 pL, but ranged from 65–110 pL as determined by measuring the diameter of droplets injected into halocarbon oil. Overaged or overdessicated embryos and embryos that bled a volume greater than that injected were destroyed and excluded from the results. Mock-injected embryos were treated identically to injected embryos except they were not injected.

Cuticle Analysis and Immunohistochemistry

For cuticle analysis, embryos were incubated at 18°C under oil for 2 days. After dissection from their vitelline membranes, embryos were washed, fixed, and their cuticles prepared as described (Zhang and Carthew, 1998). For immunohistochemistry, embryos were incubated at 25°C under oil after they were injected. At the appropriate stage, they were collected, fixed, devitellinized, and stained as described (Vincent and O'Farrell, 1992).

Genetics

The wild-type stock used for all injections was w^{1118}. Other stocks used were ftz^{13} (Jurgens et al., 1984), ttk^{1e11} (Xiong and Montell, 1993), wg^{1-8} (Nusslein-Volhard et al., 1984), and fz^{1}. For epistasis testing with Wg, Arm-Gal4 line 4a4b (Lawrence et al., 1996) was crossed to UAS-Wg^{ts} (Wilder and Perrimon, 1995). Arm-Gal4 line 4a4b is the strongest known line of the Arm-Gal4 drivers that cause uniform reporter gene expression in embryos (Lawrence et al., 1996). After syncytial blastoderm embryos were injected, they were incubated at 17°C until cuticles were collected. This temperature is fully permissive for Wg^{ts} activity and generates a naked cuticle phenotype indistinguishable from wild-type UAS-Wg (Lawrence et al., 1996). The egg-laying and injection procedures were done at room temperature, since *wg* activity is not required until a couple of hours later in embryogenesis (Bejsovec et al., 1991). While the majority of mock-injected Arm-Gal4/UAS-Wg^{ts} larvae were completely missing ventral denticles (78 of 101), the remaining individuals had one or more segments with a variable number of denticles. This variable phenotype was also observed by Lawrence et al. (1996). Since an average of 28% of injected embryos survive to secrete cuticle, it was formally possible that injection of *fz* and *Dfz2* RNA was selecting against Arm-Gal4/UAS-Wg^{ts} survivors with completely naked cuticle. Three observations argue against this possibility. First, the rate of survival for Arm-Gal4/UAS-Wg^{ts} embryos when mock injected was 42% (64 of 151), compared to 26% (62 of 240) when injected with dsRNA. This difference in survival rate would not be sufficient to account for the observed difference in cuticle phenotypes between mock- and dsRNA-injected groups. Second, individuals who had ventral denticles after being injected with dsRNA had significantly more rows of denticles than mock-injected individuals who had ventral denticles. Third, some individuals injected with dsRNA had ectopic denticle rows, which was never observed in mock-injected larvae.

For epistasis testing with *zw3*, $zw3^{M11\text{-}1}$ mutant embryos were derived from females with mosaic germ lines generated by the FLP-DFS technique as described (Siegfried et al., 1992). Mosaic females

were mated to *zw3*+ males. Since *zw3* is on the X chromosome, embryos inheriting a paternal X chromosome are partially rescued for the *zw3* phenotype (Siegfried et al., 1992). This includes partial segmentation and formation of denticle rows. We observed a predicted 1:1 ratio of null and partially rescued phenotypes in pilot experiments using standard embryo collection procedures. However, when mutant embryos were mock injected and raised under oil, a 5:1 ratio of null and rescued phenotypes was observed. We interpret this to mean that the stress of the procedure was suppressing the rescued *zw3* mutant phenotype. The epistasis test was performed twice in two different ways. The first test used w^{1118} males to generate mutant embryos. These embryos were either mock injected or injected with *fz* and *Dfz2* dsRNA and raised under oil. Cuticles of both null and paternally rescued survivors were mounted and analyzed together. There was no statistical difference in either the frequency of animals with the null phenotype or the number of denticle rows in animals with the rescued phenotype between mock-injected and dsRNA-injected cohorts (see Table 1). The second epistasis test used FM7, *ftz-lacZ*/Y males to generate mutant embryos. These embryos were injected and raised under oil as in the first test. Unhatched embryos with secreted cuticle were dissected out of their vitelline membranes, washed in heptane, and fixed in heptane:5% glutaraldehyde in PBS (1:1) for 15 min. Each embryo was washed in PBS and its anterior end was dissected off. Embryos were then stained for X-Gal activity to detect the paternally rescued embryos, and they were sorted according to null and rescued genotypes. Cuticle preparation and mounting were as described above. There was no difference in the null class or the rescued class of embryos between mock-injected and dsRNA-injected cohorts ($N \geq 14$ for each class/cohort).

Acknowledgments

We wish to thank E. Siegfried and the Bloomington Stock Center for fly stocks; I. Duncan and DSHB for antibodies; P. Adler, H. Krause, Z. C. Lai, and P. MacDonald for plasmids; and J. Franzen and C. Peebles for help with RNA hyperchromicity measurements. Thanks to Andy Fire and Lucy Xu for comments on the manuscript. This work was in part supported by the NIH (R01 EY10111). R. W. C. is a Pew Scholar in the Biomedical Sciences.

Received October 20, 1998; revised November 25, 1998.

References

Adler, P.N., Vinson, C., Park, W.J., Conover, S., and Klein, L. (1990). Molecular structure of *frizzled*, a *Drosophila* tissue polarity gene. Genetics *126*, 401–416.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1993). Current Protocols in Molecular Biology. (Pittsburgh, PA: Greene Publishing Assoc., Inc. and John Wiley and Sons, Inc.).

Axelrod, J.D., Matsuno, K., Artavanis Tsakonis, S., and Perrimon, N. (1996). Dishevelled mediates interaction between Wingless and Notch signaling pathways. Science *271*, 1826–1832.

Axelrod, J.D., Miller, J.R., Shulman, J.M., Moon, R.T., and Perrimon, N. (1998). Differential recruitment of Dishevelled provides signaling specificity in the planar cell polarity and Wingless signaling pathways. Genes Dev. *12*, 2610–2622.

Baker, N.E. (1988a). Transcription of the segment-polarity gene wingless in the imaginal discs of *Drosophila*, and the phenotype of a pupal-lethal *wg* mutation. Development *102*, 489–497.

Baker, N.E. (1988b). Embryonic and imaginal requirements for *wg*, a segment polarity gene in Drosophila. Dev. Biol. *125*, 96–108.

Bejsovec, A., and Martinas Arias, A. (1991). Roles of *wingless* in patterning the larval epidermis of Drosophila. Development *113*, 471–485.

Bejsovec, A., and Wieschaus, E. (1993). Segment polarity gene interactions modulate epidermal patterning in *Drosophila* embryos. Development *119*, 501–517.

Bhanot, P., Brink, M., Samos, C.H., Hsieh, J.-C., Wang, Y.S., Macke, J.P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the *frizzled* family from *Drosophila* functions as a *wingless* receptor. Nature *382*, 225–230.

Brunner, E., Peter, O., Schweizer, L., and Basler, K. (1997). Pangolin encodes a Lef-1 homologue that acts downstream of Armadillo to transduce the Wingless signal in *Drosophila.* Nature *385*, 829–833.

Cadigan, K.M., and Nusse, R. (1997). Wnt signaling: a common theme in animal development. Genes Dev. *11*, 3286–3305.

Cadigan, K.M., Fish, M.P., Rulifson, E.J., and Nusse, R. (1998) Wingless repression of *Drosophila frizzled 2* expression shapes the Wingless morphogen gradient in the wing. Cell *93*, 767–777.

Couso, J.P., and Martinas Arias, A. (1994). *Notch* is required for *wingless* signaling in the epidermis of Drosophila. Cell *79*, 259–272.

DiNardo, S., Sher, E., Heemskerk-Jongens, J., Kassis, J.A., and O'Farrell, P.H. (1988). Two-tiered regulation of spatially patterned *engrailed* gene expression during *Drosophila* embryogenesis. Nature *322*, 604–609.

Fire, A., Xu, S., Montgomery, M., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature *391*, 806–811.

Gubb, D., and Garcia-Bellido, A. (1982). A genetic analysis of the determination of cuticular polarity during development in *Drosophila melanogaster*. J. Embryol. Exp. Morph. *68*, 37–57.

Guo, M., Bier, E., Jan, L.Y., and Jan, Y.N. (1995). *tramtrack* acts downstream of *numb* to specify distinct daughter cell fates during asymmetric cell divisions in the *Drosophila* PNS. Neuron *14*, 913–925.

Hafen, E., Kuroiwa, A., and Gehring, W.J. (1984). Spatial distribution of transcripts from the segmentation gene *fushi tarazu* during *Drosophila* embryonic development. Cell *37*, 833–841.

Hays, R., Gibori, G.B., and Bejsovec, A. (1997). Wingless signaling generates pattern through two distinct mechanisms. Development *124*, 3727–3736.

Heberlein, U., Borod, E.R., and Chanut, F. (1998). Dorsoventral patterning in the Drosophila retina by *wingless*. Development *125*, 567–577.

Heemskerk, J., DiNardo, S., Kostriken, R., and O'Farrell, P.H. (1991) Multiple modes of *engrailed* regulation in the progression towards cell fate determination. Nature *352*, 404–410.

Heldens, J.G., Kester, H.A., Zuidema, D., and Vlak, J.M. (1997). Generation of a p10-based baculovirus expression vector in yeast with infectivity for insect larvae and insect cells. J. Virol. Methods *68*, 57–63.

Jarman, A.P., Grau, Y., Jan, L.Y., and Jan, Y.N. (1993). *atonal* is a proneural gene that directs chordotonal organ formation in the *Drosophila* peripheral nervous system. Cell *73*, 1307–1321.

Jurgens, G., Wieschaus, E., Nusslein-Volhard, C., and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. Roux's Arch. Dev. Biol. *193*, 283–295.

Klingensmith, J., and Nusse, R. (1994). Signaling by Wingless in *Drosophila*. Dev. Biol. *166*, 396–414.

Krasnow, R.E., and Adler, P.N. (1994). A single *frizzled* protein has a dual role in tissue polarity. Development *120*, 1883–1893.

Lawrence, P.A., Sanson, B., and Vincent, J.P. (1996) Compartments, *wingless* and *engrailed*: patterning the ventral epidermis of Drosophila embryos. Development *122*, 4095–4103.

MacDonald, P.M., Ingham, P., and Struhl, G. (1986). Isolation, structure, and expression of *even-skipped*: a second pair-rule gene of Drosophila containing a homeobox. Cell *47*, 721–734.

Martinas Arias, A., Baker, N., and Ingham, P. (1988). Role of the segment polarity genes in the definition and maintenance of cell states in the *Drosophila* embryo. Development *103*, 157–170.

Miklos, G.L., and Rubin, G.M. (1996). The role of the genome project in determining gene function: insights from model organisms. Cell *86*, 521–529.

Montgomery, M.K., and Fire, A. (1998). Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression. Trends Genet. *14*, 255–258.

Noordermeer, J., Klingensmith, J., Perrimon, N., and Nusse, R.

(1994). *dishevelled* and *armadillo* act in the *wingless* signaling pathway in *Drosophila*. Nature *367*, 80–83.

Nusslein-Volhard, C., Wieschaus, E., and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. Roux's Arch. Dev. Biol. *193*, 267–282.

Read, D., and Manley, J.L. (1992). Alternatively spliced transcripts of the *Drosophila tramtrack* gene encode zinc finger proteins with distinct DNA binding specificities. EMBO J. *11*, 1035–1044.

Reifegerste, R., Ma, C., and Moses, K. (1997). A polarity field is established early in the development of the *Drosophila* compound eye. Mech. Dev. *68*, 69–79.

Rubin, G.M., and Spradling, A.C. (1982). Genetic transformation of Drosophila with transposable element vectors. Science *218*, 348–353.

Scott M.P., Weiner, A.J., Hazelrigg, T.I., Polisky, B.A., Pirrotta, V., Scalenghe, F., and Kaufman, T.C. (1983). The molecular organization of the Antennapedia locus of *Drosophila*. Cell *35*, 763–776.

Siegfried, E., Chou, T.B., and Perrimon, N. (1992). *wingless* signaling acts through *zeste-white 3*, the Drosophila homolog of glycogen synthase kinase-3, to regulate engrailed and establish cell fate. Cell *71*, 1167–1179.

Siegfried, E., Wilder, E.L., and Perrimon, N. (1994). Components of wingless signaling in *Drosophila*. Nature *367*, 76–80.

Struhl, G., and Basler, K. (1993). Organizing activity of wingless protein in Drosophila. Cell *72*, 527–540.

Tomlinson, A., Strapps, W.R., and Heemskerk, J. (1997). Linking Frizzled and Wnt signaling in *Drosophila* development. Development *124*, 4515–4521.

van de Wetering, M., Cavallo, R., Dooijes, D., van Beest, M., van Es, J., Loureiro, J., Ypma, A., Hursh, D., Jones, T., Bejsovec, A., et al. (1997). Armadillo coactivates transcription driven by the product of the *Drosophila* segment polarity gene dTCF. Cell *88*, 789–799.

Vincent, J-P., and O'Farrell, P.H. (1992). The state of *engrailed* expression is not clonally transmitted during early Drosophila development. Cell *68*, 923–931.

Wilder, E.L., and Perrimon, N. (1995). Dual functions of *wingless* in the Drosophila leg imaginal disc. Development *121*, 477–488.

Xiong, W.C., and Montell, C. (1993). *tramtrack* is a transcriptional repressor required for cell fate determination in the *Drosophila* eye. Genes Dev. *7*, 1085–1096.

Zecca, M., Basler, K., and Struhl, G. (1996). Direct and long-range action of a *wingless* morphogen gradient. Cell *87*, 833–844.

Zhang, J., and Carthew, R.W. (1998). Interactions between Wingless and DFz2 during *Drosophila* wing development. Development *125*, 3075–3085.

Zheng, L., Zhang, J., and Carthew, R.W. (1995). *frizzled* regulates mirror-symmetric pattern formation in the *Drosophila* eye. Development *121*, 3045–3055.