Dfrizzled-3, a new *Drosophila* Wnt receptor, acting as an attenuator of Wingless signaling in *wingless* hypomorphic mutants

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SUMMARY

In *Drosophila*, two Frizzled proteins, Frizzled and Dffrizzled-2, have been reported to serve as receptors of Wingless. Here, we identified the third member of the *Drosophila* Frizzled family (Dfrizzled-3). In contrast to Dffrizzled-2, Dfrizzled-3 was transcriptionally upregulated by Wingless signaling. Although Dfrizzled-3 was capable of binding to Wingless in vitro, Wingless-dependent Armadillo/\(\beta\)-catenin stabilization occurred much less effectively in *Drosophila* cells transfected with Dfrizzled-3 than in those with Dffrizzled-2. Flies lacking Dfrizzled-3 activity were viable and fertile, with few morphological defects. Genetic and immunochemical analysis indicated that the absence of Dfrizzled-3 activity suppresses the effects of hypomorphic *wingless* mutations such as failure of wing and antenna formation and restores target gene expression to the normal levels without change in *wingless* expression. Wingless signaling may thus be attenuated by Dfrizzled-3 at least in *wingless* hypomorphic mutants.

Key words: *Drosophila*, frizzled, Dfz2, Dfz3, wingless, Signal attenuation

INTRODUCTION

Wnt signaling is essential for many developmental processes in both vertebrates and invertebrates (for review, see Klingensmith and Nusse, 1994; Cadigan and Nusse, 1997). In *Drosophila*, a segment polarity gene, *wingless* (*wg*), encodes a secretory Wnt protein functioning as an inductive signal for embryonic and imaginal disc development. Secreted Wg binds to its receptor on the surface of the signal-receiving cell, and activates Dishevelled (Dsh), which in turn prevents Shaggy/Zeste-White-3 from phosphorylating Armadillo (Arm)/\(\beta\)-catenin for its stabilization (Riggleman et al., 1990; Peifer et al., 1994; Yanagawa et al., 1995). The hypophosphorylated Arm forms a complex with Pangolin/dTCF, which then moves to the nucleus to activate target genes (Brunner et al., 1997; Reise et al., 1997; van de Wetering et al., 1997).

*Drosophila* Frizzled 2 (Dfz2), a member of the Frizzled (\(Fz\)) family of seven-transmembrane-domain proteins, is capable of binding to Wg and stabilizing Arm in vitro and hence, appears a Wg receptor (Bhanot et al., 1996). The overexpression of wild-type and dominant-negative forms of Dfz2 in wing imaginal discs strongly supports this scheme (Cadigan et al., 1998; Zhang and Carthew, 1998). Recent experiments using double-stranded (ds) RNA interference and a deletion uncovering Dfz2 and several adjacent genes have further clarified roles of Dfz2 in Wg signaling (Bhat, 1998; Kennerdell and Carthew, 1998; Müller et al., 1999). Kennerdell and Carthew (1998) showed the dsRNA interference of *fz* and Dfz2 genes together to produce embryonic patterning that mimics loss of *wg* function and that this patterning is not to be affected by interference of either gene alone. Bhat (1998) identified a Dfz2 deletion mutant and suggested that loss of either *fz* or Dfz2 affects the same set of neurons as those affected by loss of *wg*. While these defects are partially penetrant in embryos lacking *fz* or Dfz2, the penetrance is significantly enhanced in embryos lacking both. Defects due to the deletion mutant were partially rescued by the expression of a Dfz2 transgene. These findings appear consistent with the notion that *fz* and Dfz2 serve as redundant Wg receptors in embryos. This, however, would not necessarily mean that Fz and Dfz2 generally serves as Wg receptors. Indeed, loss- and gain-of-function analysis of Fz has indicated that Fz has no detectable role in Wg signaling required for morphogenesis other than planar polarity in imaginal discs (Cadigan et al., 1998; Zhang and Carthew, 1998).

Dfz2 may be crucial not only for response of cells to Wg signals but for the normal shape of the Wg morphogen gradient as well. According to Cadigan et al. (1998), Dfz2 expression is generally repressed by Wg signaling and, at least in the wing pouch, high levels of Dfz2 stabilize Wg molecules. Thus, in the wing pouch, a gradient of decreasing Wg stability moving toward the Wg source (dorsalventral compartment border cells) is likely to be formed. Wg activity near Wg sources is consequently reduced considerably, while Wg is allowed to reach cells far from its source.
Here, we identified the third member of Drosophila fz family, Drosophila frizzled-3 (Dfz3). Dfz3 is expressed in late embryos and imaginal discs and encodes a Wg receptor whose signal transducer activity is much less efficient than Dfz2. In contrast to Dfz2, Dfz3 expression is positively regulated by Wg signaling. The absence of Dfz3 suppressed the effects of hypomorphic wg mutants and restored target gene expression to wild-type levels without change in wg expression. Dfz3 may thus serve as an attenuator of Wg signaling.

MATERIALS AND METHODS

Fly stocks and genetics

J29 is an enhancer trap line. Dfz3G10, a null mutant of Dfz3, is a derivative of J29 (see the text). wg mutant alleles, Gal4 lines and UAS lines used are: wg1 (Baker, 1988), wgCX3, wgCX4 (Baker, 1987), wg1en (Kassis et al., 1992), ap28 (Wilson, 1981), en-Gal4 (Tabata et al., 1995), ptc-Gal4 (Johnson et al., 1995), C765-Gal4 (Zecca et al., 1996), UAS-wg6 (Wild and Perrimon, 1995) and UAS>CD2,y>flu-Darm (Zecca et al., 1996). Flies with UAS-Dfz3 were generated by P-mediated germline transformation. ptc-Gal4/UAS-wg6 flies were usually raised at 25°C. The active form of wg6 was expressed by shifting raising temperature from 25 to 16.5°C 24 hours before dissection. Darm clones were generated in J29-lacZ/ywflp; UAS>CD2,y>flu-Darm+/C765-Gal4+ flies by a 30 minute heat shock at 34°C, and clones were identified by staining with anti-Flu antibody. wg1en/wgCX3 males were obtained by crossing wg1en/Gla Bc females and wgCX3/Gla Bc males, while Dfz3G10, wg1en/wgCX3 males, by crossing Dfz3G10, wgCX3/Gla Bc females and wg1en/Gla Bc males.

Molecular cloning of Dfz3

A 550 bp DNA fragment near the P insertion was rescued by PCR and used as a probe for screening genomic DNA libraries. J29 (Dfz3) gene fragments were identified by in situ hybridization to wing discs. cDNA clones were isolated from plasmid cDNA (Brown and Kafatos, 1988) and ZAP random primer cDNA libraries. RT-PCR and 5′RACE cDNA clones were also isolated and used for cDNA structure analysis. RNA extracted from cultured cell lines were examined by RT-PCR. Primers used were: 5′TGCAAGTGTGCAAGTAGATGAGC and 5′AG-GATCCCAACCCATCATCGAAAGCAC for fz RNA detection, 5′CTCGAGGTTCGACGTCATCGAAGC and 5′CAGTTTTTTTGAATGCTCCGCGGC for Dfz2 RNA detection, and 5′GG-TCCGGTTTCAGCTCTTGGAGT and 5′CTGATTGGTTGTGAC-CTGCCAGGTT for Dfz3 RNA detection.

**Fig. 1.** Similarity in Dfz3 and wg expression patterns (A-K) and requirements of Wg signaling activity for Dfz3 expression (L-S) in the imaginal discs. In all pictures, anterior is left and dorsal is up. J29 (Dfz3)-lacZ expression is always colored in green. All discs were prepared from late third-instar larvae. Thin line, dorsal (D) and ventral (V) compartment border; wp, wing pouch; n, notum. (A-H), Wing (A-D) and leg (E-H) discs. (A,E, green) J29 (Dfz3)-lacZ expression; (B,F, red) Wg expression; (C,G) merged pictures; (D,H) in situ hybridization. Note that J29 (Dfz3) expression is similar to but occupies a much wider area than wg expression. (H) Ventral restriction of Dfz3 RNA expression; (I,J) Dfz3 antisense RNA hybridization to and wg-lacZ expression in eye-antennal discs, respectively. The inset shows strong and moderate expression of Dfz3-lacZ in photoreceptors R8 (arrowhead) and R7 (arrow), respectively. (K) The absence of Dfz3 RNA expression in Dfz3G10leg discs. (L-O) Wg signaling is necessary and sufficient for Dfz3 expression. (L) wgCX3 leg discs were stained for wg-lacZ (brown) and a distal structure marker, BarH1 (black; T. K., unpublished data). (M) As with wg-lacZ expression (see L), Dfz3-lacZ expression (β-galactosidase activity staining, blue) was reduced in size in wgCX3 mutants. Note that reduction of the Wg-expressing area causes ectopic axis formation (see two arrowheads in L). (N) Dfz3-lacZ misexpression (see the white arrowhead) induced by ptc-Gal4 driven UAS-wg6. Red, Wg expression. (O) Misexpression of Dfz3-lacZ in dorsal flip-out Darm clones detected by anti-Flu antibody (red). (P–S) Complementary expression of Dfz2 and Dfz3 in leg (P–R) and wing (S) discs. Red, Dfz2 RNA. (R) Merged picture of P (Dfz3-lacZ) and Q (Dfz2 RNA). Scale bar in D: 65 μm for A–D, S; 80 μm for E–H, L–R; 77 μm for I, J; 60 μm for K.
Antibodies and tissue staining

Immunohistochemistry and in situ hybridization were carried out as described previously (Sato et al., 1999). Simultaneous staining of discs for protein and RNA was carried out using an original protocol (Goto and Hayashi, 1997) modified as follows. Fixed discs were prepared as described (Sato et al., 1999) and then treated with 0.5% NP-40 in PBTH for 30 minutes at room temperature. After antibody staining and the second fixation, discs were treated with Protease K for 1-4 minutes at room temperature. Protease reaction was stopped by adding 2 mg/ml glycine in PTw. Postfixation was carried out in 4% paraformaldehyde and 0.2% glutaraldehyde/PBS for 20 minutes. Hybridization solution was replaced with 20 mM Tris-HCl (pH 8.0) containing 2.5 mM EDTA, 300 mM NaCl, 1× Denhard’s solution, 1 mg/ml tRNA and 50% formamide. Dfz2 and Dfz3 anti-sense RNA probes, labeled with digoxigenin-11-UTP, were prepared by in vitro transcription using as templates a 2.3 kb KpnI-NotI fragment of Dfz3 cDNA and a 2.5 kb XhoI-SpeI fragment of Dfz2 cDNA (Bhanot et al., 1996), respectively. Antibodies used were: rabbit anti-β-gal (1:2000 dilution for DAB staining; Cappel), mouse anti-β-gal (1:500; Promega), anti-BarH1 (Higashijima et al., 1992), anti-Wg (Brook and Cohen, 1996), anti-Dac (Mardon et al., 1994), anti-Arm (Peifer, 1993), anti-α-catemin (Oda et al., 1993), anti-Dll (Vachon et al., 1992), mouse anti-Flu (1:100; Babco), goat anti-rabbit biotin conjugated (1:500; VECTOR), goat anti-mouse biotin conjugated (1:500; VECTOR), anti-mouse Cy3 conjugated (1:100; Amersham), anti-rabbit Cy3 conjugated (1:50; Jackson), avidin FITC conjugated (1:50; PIERCE), anti-mouse alkaline phosphatase conjugated (1:2000; Promega), anti-rat alkaline phosphatase conjugated (1:2000; Promega) and anti-digoxigenin alkaline phosphatase conjugated (1:2000; Roche) antibodies. Five times higher concentrations of antibodies were used for fluorescence staining.

Cell culture, Wg binding assay and Arm stabilization assay

pMK33 containing a Dfz2 cDNA insert (pMK33-Dfz2) and S2 cells were obtained from R. Nusse and S. Yanagawa (Bhanot et al., 1996). pMK33-Dfz3 was constructed by replacing the Dfz2 sequence with the counterpart of Dfz3. In these constructs, Dfz2 and Dfz3 transcription is under the control of the metallothionein promoter. pMK33-Dfz2 and pMK33-Dfz3 were used for both transient expression and generation of stably transfected cell lines. S2 cells stably expressing Dfz2 (S2/Dfz2-C1-4) and Dfz3 (S2/Dfz3-C1-4) along with those stably possessing pMK33 (S2/pMK-C1-4) were obtained by the calcium phosphate precipitation method. Clonal cell lines were obtained as described previously (Saigo et al., 1983). Dfz2 and Dfz3 were transiently expressed using CellFECTIN (Gibco) according to manufacturer’s protocol. Wg-containing conditioned medium was prepared as Fig. 2.
described previously (Bhanot et al., 1996). Wg-binding, Wg-detection and Arm stabilization were carried out according to Bhanot et al. (1996).

RESULTS

Identification of Dfz3, a new Drosophila fz family member

Genes whose expression is under the control of Wg signaling may exhibit expression patterns similar to those of wg. Enhancer trap line analysis led us to the discovery of a trap line, J29, that exhibits wg-like reporter gene (lacZ) expression in wing discs (Fig. 1A,B). Relevant genomic DNA and cDNA clones were isolated using PCR rescue followed by library screening, RT-PCR and 5' RACE (see Fig. 2A). Nucleotide sequence analysis and in situ hybridization (see below) disclosed the trapped gene (J29 gene) to encode a polypeptide with domain structure and amino acid sequence similar to those of Dfz2 (Bhanot et al., 1996) and Fz (Vinson et al., 1989). Thus, the J29 gene is referred to hereafter as Dfizzled-3 (Dfz3). Dfz3 is situated at 1C1 on the first chromosome (data not shown).

The predicted RNA start of Dfz3 is 5'TTCAGTTT (Hultmark et al., 1986) and J29-P insertion was found to occur several bp upstream of the RNA start (Fig. 2A). Dfz3 is a TATA-less gene and associated with 5'GTCG, a downstream-promoting-element-like element (Burke and Kadonaga, 1997) at about +30 bp (Fig. 2A). Total cDNA length was estimated at 2.2 kb, which agrees well with 2.3 kb RNA observed in northern blots (data not shown). Dfz3 contains two exons that may code for a polypeptide of 581 amino acid residues (Fig. 4C-E). Dfz3 is situated at 1C1 on the first chromosome (data not shown).

Similarity in expression patterns of Dfz3 and wg

Using reporter gene expression and in situ hybridization, Dfz3 expression was examined. Dfz3 RNA expression was essentially identical to that of Dfz3-lacZ. Staining for Wg and Dfz3-lacZ indicated Dfz3 expression to be similar to but to occupy a much wider area than wg expression (Fig. 1A-J), suggesting that Dfz3 is a general target gene of Wg signaling. As with Wg signals, strong Dfz3-lacZ expression signals were detected along the dorsoventral compartment border of the late-third-instar wing pouch and in future hinge and notum regions (Figs 1A, 3E). In the wing pouch, Wg is present at high levels in cells expressing wg RNA, but drops off sharply on moving away from the stripe (Couso et al., 1994; Cadigan et al., 1998). Consistent with this, weak broad Dfz3-lacZ expression was detected in most wing pouch cells expressing Distal-less (Dll), a target gene of Wg signaling (Fig. 3D-F; Couso et al., 1994) in addition to strong reporter gene expression along the dorsoventral compartment border. Coexpression of Dfz3 and Bar homeobox genes (Kojima et al., 1991; Sato et al., 1999) was detected in the lateral prescutum (Fig. 3A-C). Bar expression in the lateral prescutum was previously shown to be positively regulated by Wg produced in scutum cells (Sato et al., 1999).

In leg discs, wg is expressed in anteroventral cells along the anteroposterior compartment border and required for fate determination of both dorsal and ventral cells (Wilder and Perrimon, 1995; Brook and Cohen, 1996; Jiang and Struhl, 1996). At early third instar, Dfz3 expression was evident in both ventral and dorsal cells within about 6 cell diameters from the Wg sources (Fig. 3G-I). Note that Dfz3 expression considerably differs from that of H15, an enhancer trap of a leg-specific gene whose expression is positively and negatively regulated by Wg and Decapentaplegic (Dpp) signaling, respectively (Fig. 3K-M; Brook and Cohen, 1996). At late third instar, Dfz3 expression in leg discs was ventrally restricted, as noted for H15 (Fig. 3J,N). Late Dfz3 expression should thus be negatively regulated by a dorsal factor.

In late-third-instar eye discs, wg is expressed in undetermined cells along the periphery of the eye disc anterior to the morphogenetic furrow (Fig. 1J; Ma and Moses, 1995). Dfz3 RNA was detected in cells within several cell diameters from Wg sources (Fig. 1I). Although no Wg is expressed in ommatidial cells, Dfz3-lacZ expression was detected strongly and considerably in R8 and R7 photoreceptors (inset of Fig. 1I), respectively; Dfz3-lacZ was also expressed in other photoreceptors at later stages. Ommatidial Dfz3 expression may not be related to Wg signaling.

Although Wg accumulates in a striped pattern in early embryos, neither the ectoderm nor CNS of early embryos were associated with Dfz3 expression (compare Fig. 4A and B), indicating that Dfz3 is unrelated to early Wg stripes. Striped Dfz3 expression could initially be seen in ventral ectoderm at later stages (Fig. 4C,D). Similar delayed striped expression has also been seen in DWnt4 (Fig. 4F; Graba et al., 1995). As with wg (Fig. 4B, Baker, 1987), Dfz3 was expressed strongly in brain, proventriculus, parasegment (PS) 8 of the visceral mesoderm (VM-PS8), Malpighian tubules and anal plate (Fig. 4E).

DWnt4 maps close to wg and they are coexpressed in many cells, although their expression differs in several respects (Graba et al., 1995). wg is specifically expressed in Malpighian tubes, while DWnt4 but not wg is expressed in the nerve cord, VM-PS4 and the dorsal edge of an embryo. Dfz3 was expressed in the dorsal edge of the embryo (Fig. 4C-E) but not in the nerve cord and VM-PS4.

Upregulation of Dfz3 expression by Wg signaling

To determine whether Wg signaling is required for Dfz3 expression, examination was made of the effects of reduction and misexpression of Wg signals on Dfz3-lacZ expression. On a wg hypomorphic mutant (wg<sup>cx5</sup>) background, the area of Dfz3-lacZ expression in leg discs decreased with a reduction of Wg expression (compare Fig. 1L and 1M). All embryonic Dfz3 expression other than that occurring along the dorsal edge and weak expression in brain disappeared in a wg null mutant, wg<sup>cx4</sup> (Fig. 4G). When UAS-wg<sup>ts</sup> was driven by ptc-Gal4, Dfz3-lacZ misexpression occurred in anterior cells along the anteroposterior compartment border in a cell-non-autonomous fashion (Fig. 1N). Similar but cell-autonomous misexpression of Dfz3-lacZ was noted in flip-out clones expressing DeltaArm, a constitutively active form of Arm (Fig. 1O; Zecca et al., 1996). From these findings and expression of Dfz3-lacZ (see Figs 1A-
J and 4C-E), we conclude that Dfz3 expression is positively regulated by Wg signaling, which gives the opposite effect on Dfz2 expression (Cadigan et al., 1998). Consistent with this conclusion, at least in leg and wing discs, Dfz2 and Dfz3 showed virtually complementary expression (Fig. 1P-S).

That Dfz3 expression along the dorsal edge of an embryo where DWnt4 is expressed (Graba et al., 1995) is insensitive to the absence of wg activity (Fig. 4G) suggests that dorsal-edge Dfz3 expression may be due to DWnt4 signaling.

**Dfz3 as a Wnt receptor with inefficient Wg signal transducer activity in cultured S2 cells**

To determine whether Dfz3 is capable of binding to Wg and transducing Wg signals, Dfz2 and Dfz3 were expressed under the control of the metallothionein promoter in Schneider line 2 (S2) cells (Schneider, 1972). RT-PCR analysis showed that neither endogenous fz, Dfz2 nor Dfz3 are expressed in S2 cells (data not shown). To examine Wg binding, either Dfz2 or Dfz3 was transiently expressed in S2 cells. Transfectants were then incubated with Wg-conditioned medium and stained with anti-Wg antibody. Not only Dfz2- but also Dfz3-transfectants showed strong surface staining (Fig. 5B,C) while no surface signals could be detected in PMK33-transfected cells (Fig. 5A), indicating tight binding of Wg to Dfz3.

To determine whether Dfz3 is capable of transducing Wg signals, Arm stabilization in response to added Wg (Riggleman et al., 1993) was assessed. Arm was previously shown to be phosphorylated by Zeste-White 3 family fz-kinases (Yanagawa et al., 1995) was assessed. Arm was transiently expressed in S2 cells, Dfz3 is capable of serving as a transducer of Wg signals but its activity is much less than that of Dfz2.

**DNA was extracted from 108 balanced mutant stocks, digested with restriction enzymes, size fractionated with agarose gel electrophoresis and subjected to Southern blotting. Search was made for lines with abnormal mobility of Dfz3 fragments. A deletion mutant line, Dfz3G10, was obtained. In Dfz3G10, the RNA start site, the first exon and a portion of the intron of Dfz3 were deleted (Fig. 2A) and no Dfz3 RNA expression could be detected in both embryos (Fig. 4H) and imaginal discs (Fig. 1K), indicating that Dfz3G10 is a null mutation of Dfz3. Flies homozygous for Dfz3G10 were surprisingly found viable and fertile, with few appreciable morphological defects. Planar polarity was also normal (data not shown). Double knockout flies of fz (fz1) and Dfz3 were also constructed and found viable and fertile, with no morphological defects other than those in planar polarity, which are characteristic of fz homozygotes (data not shown; Park et al., 1994).**

**Dfz3 as an attenuator of Wg signaling**

Dfz3 may be involved in Wg signaling required for adult appendage formation. For clarification of this point, study was made to find possible interaction between Dfz3 and Wg signaling on various wg mutant backgrounds. Wing blades are frequently absent from flies mutant for wg (Baker, 1988). Thus, first examined was whether the wg0 phenotype is affected by the absence of Dfz3. As shown in Fig. 6A, this defect was partially rescued through the elimination of Dfz3 activity. On a wg+/wgCX4 background, fractions of flies with two wings increased from 46% to 87%, while those of one wing and wingless flies, respectively, reduced from 44% and 10% to 13% and 0.5%.

The wing-less phenotype of wg0 is enhanced in a heterozygous apterous (ap) mutant background (see Fig. 6B): no wing blade was generated at approx. 90% presumptive wing-blade-forming sites in wg0 heterozygous flies for ap. Fig. 6B showed that wing blade formation increased 2-fold in the absence of Dfz3 activity. Since wgCX4 and wg0, respectively, are null and regulatory mutant alleles (Baker, 1987), these effects are not due to possible change in Wg protein conformation. Wild-type Dfz3 may thus serve as an attenuator of Wg signaling at least in a wg hypomorphic mutant background and the absence of Dfz3 may increase Wg signaling and stimulate wing formation.

To confirm that Dfz3 attenuates Wg signaling, examination was made of the effects of Dfz3 absence in a different developmental context. Nearly all wg11en/wgCX3 flies lacked antennal structures (Fig. 6C,E). As shown in Fig. 6C,F, this antenna-less phenotype was significantly rescued by removing Dfz3 activity; not only incomplete antennal structures but also those that were complete were regenerated at more than 70% of putative antennal sites (Fig. 6C). Distal antennal segment formation requires the circular expression of Bar homeobox genes (Kojima et al., 1991; T. K., unpublished data). Dachshund (Dac) is required for the formation of proximal leg structures and expressed circularly in leg and antenna discs (Mardon et al., 1994). Thus, wg11en/wgCX3 fly discs with or without Dfz3 activity were stained for Wg, BarH1 and Dac. When there was Dfz3 activity, antennal discs were small and no or little expression of BarH1 and Dac was detected (n=18; Fig. 6H,K). In the absence of Dfz3 activity, about 10% of the discs (n=42), probably corresponding to the completely rescued type, exhibited circular BarH1 and Dac expression similar to that of
wild-type discs (Fig. 6L). In about 50% of discs, presumably corresponding to the partially rescued type, Dac expression was partially restored without recovery of BarH1 expression (Fig. 6M). In contrast to BarH1 and Dac, no Wg expression was detected in the rescued mutant discs (compare Fig. 6G and 6I), indicating that Wg expression is not enhanced by the absence of Dfz3. That wg\textsuperscript{CX3} and wg\textsuperscript{11en} are regulatory mutant alleles of Wg suggests again that the genetic interactions found here would not be due to possible change in Wg protein structure, but simply to reduction in transcription products of Wg. It thus follows that in Wg hypomorphic mutants, Dfz3 reduces Wg signaling activity required for antennal formation without changing Wg expression and, accordingly, Dfz3 would appear to function as a negative factor or attenuator of Wg signaling at least on a Wg hypomorphic mutant background.

**Fig. 3.** Dfz3 as a general Wg-signaling-target gene in imaginal discs. Dfz3-lacZ is expressed in most imaginal disc cells expressing Wg-signaling-target genes. In all pictures, anterior is left and dorsal is up. Thin white lines indicate putative dorsal (D)/ventral (V) compartment border. Green signals in A-J, Thin white lines indicate putative dorsal (D)/ventral (V) compartment signaling-target genes. In all pictures, anterior is left and dorsal is up. Dfz3 expression in A-J, Dfz3 RNA expression along the D/V border expressing Wg (see E). (G-J) Dfz3-lacZ expression in early (G-I) and late (J) third-instar leg discs. Red, Wg. Note that Dfz3-lacZ expression is not restricted ventrally in early-third-instar discs (I). (K-N) H15-lacZ expression in early (K-M) and late (N) third-instar leg discs. Red, Wg; green, H15-lacZ. Note that no H15-lacZ expression occurs in dorsal cells throughout development. Scale bar in F; 60 μm for A-C; 75 μm for D-F; 70 μm for G-I, K-M; 95 μm for J,N.

**Wg signaling transduced by high levels of Dfz2 is suppressed by low levels of Dfz3 on a Wg hypomorphic mutant condition**

As described above, Dfz2 and Dfz3 are downregulated and upregulated, respectively, by Wg signaling (see Fig. 1P-S) and Wg signaling activity in some Wg hypomorphic mutant discs is restored to levels similar, if not identical, to those of wild type upon removing Dfz3 activity (Fig. 6L). Thus, it may be suggested that, in ventral cells of Wg hypomorphic antennal discs, Dfz2 expression is de-repressed and Dfz3 expression is repressed while the de-repression of Dfz3 expression is canceled by the removal of Dfz3 activity. We tested this possibility directly by staining wild-type and mutant antennal discs for Dfz2 and Dfz3 RNA (Fig. 6N-S). As anticipated, Dfz2 expression was de-repressed in the ventral cells of Wg\textsuperscript{11en}/Wg\textsuperscript{CX3} antennal discs (n=16; compare Fig. 6O with N), and repressed to a marginal level at least in 10% of antennal discs (n=39) upon removing Dfz3 activity (Fig. 6P) as in the case of wild type (Fig. 6N). Fig. 6R shows Dfz3 expression to reduce to a marginal level in Wg hypomorphic mutants. It may

**Fig. 4.** Dfz3-lacZ and Dfz3 RNA expression in embryos. In all pictures, anterior is left. (A,C,E) Dfz3-lacZ expression; (D,G,H) Dfz3 RNA expression. Thick arrow, dorsal edge. (A) No Dfz3-lacZ signals can be seen until stage 9. (B) A stage 9 embryo showing typical Wg-lacZ stripes. (C) Weak striped Dfz3-lacZ expression (labeled with stars) becomes discernible at stage 11. (D) Dfz3 striped expression in the ventral ectoderm is more clearly visualized by Dfz3 RNA. As can be seen in C,E, Dfz3-lacZ expression occurs in the brain (br), proventriculus (pr), Malpighian tubules (mt), anal plate (ap) and visceral mesoderm parasegment 8 (PS8). Although no Wg is expressed, Dfz3 expression is evident along the dorsal edge (thick arrow in D). (F) DWhn4 RNA expression. Dfz3 RNA expression along the dorsal edge in Wg\textsuperscript{CX3} embryos (G, see arrow) indicates that dorsal Dfz3 expression is irrelevant to Wg signaling. The much stronger Dfz3 expression in G than that in D is mainly due to differences in focal plane and staining condition. (H) No Dfz3 RNA expression occurs in Dfz3\textsuperscript{H10}. Scale bar in H, 100 μm.
Fig. 5. (A-C) Wg binding, (D) fz family RNA expression and (E) Arm stabilization in cultured cells. (A-C) Strong surface stainings with anti-Wg antibody are evident in transfectants with Dfz2 (B) and Dfz3 cDNA (C) (see arrows), while no surface signals can be seen in pMK33-transfected cells (A). (D) fz, Dfz2 and Dfz3 RNA expression in S2/pMK-C3, S2/Dfz2-C3 and S2/Dfz3-C3 cells was analyzed by RT-PCR. RNA was extracted from corresponding Drosophila cells after 0-16 hour copper (Cu) incubation. No or little fz RNA expression can be seen in all cell lines examined. Note that S2/Dfz2-C3 and S2/Dfz3-C3, respectively, express Dfz2 and Dfz3 RNA specifically, while neither Dfz2 nor Dfz3 RNA expression can be detected in S2/pMK-C3. (E) Lanes 1-4, S2/pMK-C3; lanes 5-8, S2/Dfz2-C3; lanes 9-18, S2/Dfz3-C1-4. α-catenin was used as a control for western blotting. The presence or absence of Wg and 16-hour copper incubation are shown in the lower margin by ‘+’ or ‘-’. Strong Wg-dependent Arm stabilization in S2/Dfz2-C3 is evident, while Wg-dependent Arm stabilization occurs at a marginal level (C1,C2) or at a considerably low but significant level (C3,C4). As described previously (Bhanot et al., 1996), copper induction did not increase Arm stabilization (compare lanes 6 and 8). Scale bar in C; 15 μm for A-C.

Fig. 6. Suppression of wing-less and antenna-less phenotypes of wg hypomorphic mutants by the absence of the Dfz3 activity. (A-C) Numerical data of the effects of the absence of Dfz3 activity on wing formation in wg1/wgCX3 (A) and wg1/wg1 ap7‌ (B) flies and antenna formation in wg1/en/wgCX3 flies (C). 0W, Wing-less flies; 1W, flies possessing only 1 wing; 2W, normal flies. (D-F) Adult antenna morphologies are shown in D (wild type), E (mutant; antenna-less) and F (completely and partially rescued antenna). Black arrowheads show completely rescued or wild-type antenna; white arrowheads, the absence of antenna; grey arrowhead, partially rescued antenna; arrow, arista. (G-I) Antenna disc stained for Wg (red, arrowhead) and BarH1 (green, arrow). (G) Wild type. Circular BarH1 and ventral Wg expression are clearly seen. (H) wg1/en/wgCX3 mutant. Neither BarH1 nor ventral Wg expression can be seen in antenna primordia. (I) Completely rescued antenna disc. Circular BarH1 expression is evident, but no Wg signals can be detected. (J-M) Antenna disc stained for BarH1 (green, arrow) and Dac (red, arrowhead). (J) Wild type. Circular Dac expression can be seen outside the BarH1 ring. (K) wg1/en/wgCX3 mutant. Neither BarH1 nor Dac expression can be seen in antenna primordia. (L) A completely rescued antenna disc. Simultaneous expression of BarH1 and Dac is evident. (M) A partially rescued antenna disc showing dotted Dac expression (arrowhead). (N-P) Dfz2 and (Q-S) Dfz3 RNA expression. The ventral side of antennal discs is shown by arrows. (N) Dfz2 expression in wild-type eye-antennal disc. Dfz2 expression is repressed in ventral antennal disc cells under the influence of strong Wg signaling. (O) A wg1/en/wgCX3 antennal disc in which Dfz2 expression is derepressed ventrally. (P) A Dfz3G10; wg1/en/wgCX3 antennal disc in which ventral derepression of Dfz2 expression appears virtually completely restored. (Q) Dfz3 expression in a wild-type eye-antennal disc, in which Dfz3 expression is strongly upregulated ventrally. Dfz3 is expressed in the ventral (white arrowhead) and the dorsal (black arrowhead) side of eye disc. (R) Dfz3 expression in a wg1/en/wgCX3 eye-antennal disc. Dfz3 expression is significantly reduced in the ventral side (compare R with Q). Dfz3 is not expressed in the ventral side of eye disc (white arrowhead), but expressed in the dorsal side (black arrowhead). (S) A Dfz3G10; wg1/en/wgCX3 antennal disc. No Dfz3 expression occurs. Scale bar in S, 220 μm for D-F; 45 μm for G-P; S; 33 μm for Q,R.
thus follow that, at least on a wg hypomorphic mutant condition, very low levels of Dfz3 are capable of effectively suppressing Wg signaling transduced by high levels of Dfz2.

Absence of the counterpart of Dfz3 in Caenorhabditis elegans

Virtually the entire nucleotide sequence of the C. elegans genome has been determined (Ruvkun and Hobert, 1998). The C. elegans genome appears to contain four fz-members, two of which have been genetically analyzed as mom-5 and lin-17 (Sawa et al., 1996; Thorpe et al., 1997; Rocheleau et al. 1997) and one of which has been identified as Cfz1 by Wang et al. (1992). The remaining is F27E11.3, which was identified by the worm genome project (Ruvkun and Hobert, 1998). As detailed in the legend to Fig. 2, F27E11.3 cDNA sequence, inferred using a computer program, should be considerably modified. The modified version is hereafter referred to as Cfz2. Examination was made of amino acid sequence homology among Fz family members in C. elegans and D. melanogaster (Fig. 2C). While Cfz2 may be the ortholog of Dfz2, no counterpart of Dfz3 could be detected in C. elegans.

DISCUSSION

The present work indicates that Wg signal transduction pathway in Drosophila possesses a new Wg-binding protein, Dfz3, which may serve as an attenuator for Wg signaling at least on a wg hypomorphic mutant background.

Dfz3 as attenuator of Wg signaling

Wing-less and antenna-less phenotypes of wg hypomorphic mutants were partially or completely rescued by the absence of Dfz3 activity (Fig. 6). No appreciable recovery of wg expression was detected in imaginal discs, but the expression of Bar and Dac, acting downstream of Wg signaling, was remarkably restored by the absence of Dfz3 activity (Fig. 6I,L,M). Ventral Dfz2 repression in antennal discs, which is derepressed on Wg mutant backgrounds, was also restored on removing Dfz3 activity (see Fig. 6O,P). Since Dfz3 is a transmembrane protein similar in structure to Dfz2, a putative Wg receptor (Bhanot et al., 1996) and capable of binding to Wg in vitro (see Fig. 5C), Dfz3 may be a Wg receptor capable of acting as a Wg-signaling attenuator.

Antagonistic interactions have been shown to be involved in vertebrate Wnt signaling. sFRP is a CRD-like protein capable of competing with Wnt receptors for Wnt ligands (Wodarz and Nusse, 1998). WIF is a putative Wnt-binding protein presumed to suppress Wnt signaling (Hsieh et al., 1999). Thus, Dfz3-dependent attenuation of Wg signaling might be due to antagonistic interactions at the level of Wg receptors, in which Dfz3 competes with Dfz2 for Wg and possibly Dsh with consequently abortive Wg signal transduction. However, this simple antagonistic interaction model may not fully account for Dfz3-dependent suppression of Wg signaling, since (1) in wg hypomorphic mutants, only a trace of Dfz3 appeared effective enough to suppress Wg signaling transduced with high levels of Dfz2 (see Fig. 6N-S), and (2) unlike sFRP and WIF, Dfz3 overexpression gave little effects on Drosophila morphology (data not shown). Our preliminary experiments rather suggested that Dfz3 may possess some positive roles in Wg signaling, since Dfz3 overexpression caused weak misexpression of Dll, a Wg-signaling-target gene, in the future wing pouch.

Complex genetic interactions between Wnt signaling and Frizzled have been observed in endoderm specification in C. elegans (Thorpe et al., 1997; Rocheleau et al., 1997). In the absence of Mom-2 (Wnt), Mom-5 (Frizzled) appears to partially suppress redundant inputs of Wnt signaling. Thus, possible Wg/Dfz3 signaling might suppress the redundant inputs of Wg signaling or active factors that inhibit Wg signaling.

Wg signaling activity essential for normal morphogenesis may be very low

One of the most intriguing findings of the present study is that, in the absence of Dfz3 activity, very low levels of Wg, produced in wg hypomorphic mutant discs and hardly detectable with anti-Wg antibody staining, are still capable of inducing normal levels of target gene expression and repression (see Fig. 6G-S). Reduced Dfz2 expression area in the ventral antennal disc may serve as a measure of the distribution of Wg signals. Fig. 6P shows the reduced Dfz2 expression area and hence Wg signal distribution to be recovered in a significant fraction of wg hypomorphic mutant discs lacking Dfz3 activity. Thus, it might be considered that (1) only very low levels of Wg activity are essential for normal (antenal) disc development and (2) in wild type, a considerable fraction of Wg signals are either dispensable or neutralized by a negative mechanism, which may include Dfz3.

Developmental changes in components of the Wg receptor system

Recent experiments showed that fz and Dfz2 are functionally redundant to each other in embryos (Kennerdell and Carthew, 1998); fz, however, may not be involved in Wg signaling in late-embryonic and larval stages. Interestingly, Dfz3 is expressed in late embryos and imaginal discs but not early embryos. Thus, in early embryos, Fz, Dfz2 and possibly additional Fz members other than Dfz3 may form a Wg receptor system (see Bhat, 1998), while Wg receptor systems in subsequent developmental stages may be comprised of Dfz2, Dfz3 and additional Fz members other than Fz.

Dfz3 expression is intimately related to Wg expression. Dfz3 expression is possibly independent of Wg activity in two tissues, dorsal ectodermal edge of stage 11-13 embryos (see Fig. 4D,G) and differentiated photoreceptor cells (see the inset of Fig. 1). In the former, DWnt4 but not Wg is expressed (Graba et al., 1995), while no Wnt expression has been reported in the latter. In vertebrates, Wnt protein can bind to plural Fz (Bhanot et al., 1996). Dfz3 may thus interact not only with Wg but also with DWnt4 or other Drosophila Wnt proteins.

During preparation of this manuscript, we were informed by M. Boutros and M. Mlodzik that they have independently identified a fz gene situated at 1C1 on the first chromosome (personal communication).

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