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Wingless signaling in the *Drosophila* embryo: zygotic requirements and the role of the *frizzled* genes

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SUMMARY

Wingless signaling plays a central role during epidermal patterning in *Drosophila*. We have analyzed zygotic requirements for Wingless signaling in the embryonic ectoderm by generating synthetic deficiencies that uncover more than 99% of the genome.

We found no genes required for initial *wingless* expression, other than previously identified segmentation genes. In contrast, maintenance of *wingless* expression shows a high degree of zygotic transcriptional requirements. Besides known genes, we have identified at least two additional genomic regions containing new genes involved in Wingless maintenance.

We also assayed for the zygotic requirements for Wingless response and found that no single genomic region was required for the cytoplasmic accumulation of Armadillo in the receiving cells. Surprisingly, embryos homozygously deleted for the candidate Wingless receptor, *Dfrizzled2*, showed a normal Wingless response. However, the Armadillo response to Wingless was strongly reduced in double mutants of both known members of the frizzled family in *Drosophila*, *frizzled* and *Dfrizzled2*. Based on their expression pattern during embryogenesis, different Frizzled receptors may play unique but overlapping roles in development. In particular, we suggest that Frizzled and Dfrizzled2 are both required for Wingless autoregulation, but might be dispensable for late Engrailed maintenance. While Wingless signaling in embryos mutant for *frizzled* and *Dfrizzled2* is affected, Wingless protein is still internalized into cells adjacent to *wingless*-expressing cells. Incorporation of Wingless protein may therefore involve cell surface molecules in addition to the genetically defined signaling receptors of the *frizzled* family.

Key words: *Drosophila*, embryo, *frizzled*, signal transduction, *wingless*

INTRODUCTION

The cell signaling molecule Wingless (Wg) belongs to a family of secreted glycoproteins and is involved in a large variety of cell fate decisions throughout the life of *Drosophila* (Wodarz and Nusse, 1998). In the embryonic ectoderm, the segmentally repeated expression pattern of *wg* is established by pair-rule segmentation genes (Nüsslein-Volhard and Wieschaus, 1980; Siegfried and Perrimon, 1994) and is required for specification of cell fates along the anteroposterior axis of each segment (Peifer and Bejsovec, 1992). One important function of *wg* is to ensure stable expression of the homeotic selector gene *engrailed (en)* in the posterior of each segment (Bejsovec and Martinez-Arias, 1991; Hemskerk et al., 1991). In addition, *wg* expression is necessary to maintain its own expression through an autoregulatory loop (Bejsovec and Martinez-Arias, 1991; Bejsovec and Wieschaus, 1993; Li and Noll, 1993; Hooper, 1994; Yoffe et al., 1995).

Secretion of Wg requires the function of an ER resident protein encoded by the *porcupine* gene (Kadowaki et al., 1996). After binding to the cell surface, Wg is internalized and can be found in intracellular compartments characteristic of the endocytic pathway (van den Heuvel et al., 1989; Gonzalez et al., 1991; Bejsovec and Wieschaus, 1995). Candidate receptors for Wg are seven-pass transmembrane proteins of the frizzled family. In Drosophila two frizzled genes have been identified thus far: *frizzled* (*fz*) and *Drosophila frizzled2* (*Dfz2*) (Vinson et al., 1989; Bhanot et al., 1996). Both proteins exhibit molecular properties consistent with a role as Wg receptors (Nusse et al., 1997). Nevertheless the identification of the functional Wg receptor in the embryo has been hampered by the fact that mutations in *Dfz2* are not available. Loss-of-function mutations in *fz* are viable and result in a characteristic tissue polarity phenotype (Vinson and Adler, 1987). In the present paper, we present evidence that both members of the frizzled family in flies function redundantly during Wg signaling in the embryo.

As an immediate early response to Wg binding, receiving cells increase their levels of cytoplasmic Armadillo (Arm) protein, the fly homologue of vertebrate β-catenin (Riggleman et al., 1990; Peifer et al, 1994; Willert and Nusse, 1998). The accumulation of cytoplasmic Arm correlates with a change in its

phosphorylation state and appears to be based upon a modulation in Arm protein stability rather than an increase in its synthesis (van Leuven et al., 1994; Pai et al., 1997). In the embryo, activity of the maternally supplied phosphoprotein Dishevelled and the protein kinase Zeste-white 3 are required to regulate cytoplasmic Arm levels (Siegfried et al., 1992; Klingensmith et al., 1994). Arm functions in Wg signaling by binding the transcription factor *dTCF/pangolin* and acting as a transcriptional coactivator (Brunner et al., 1997; van de Wetering et al., 1997).

In the present paper, we have analyzed the zygotic transcriptional requirements for the following aspects of Wg signaling in the embryo: (1) initial expression of *wg*, (2) maintenance of *wg* expression and (3) accumulation of Arm in response to Wg. We found that most alterations in *wg* expression can be attributed to deletion of previously known loci involved in patterning. In addition, two genomic regions are required for certain aspects of *wg* maintenance and cannot be explained by known loci. While transduction of the Wg signal did not require a single genomic region zygotically, we found that double mutants of two members of the frizzled family, *fz* and *Dfz2*, show strong reduction in Wg signaling. We further show that internalization of Wg occurs in the absence of functional Wg signaling receptors. This result is supported by the identification of a genomic region needed in the embryo for transport of Wg protein by neighboring cells. The results of our analysis indicate that in addition to the known genes involved in Wg signaling, there are only a few more genes that can be identified based on their zygotic loss of function phenotype.

MATERIALS AND METHODS

Antibodies

The rat anti-Wg antibody and the anti-Dfz antibody was provided by R. Nusse (HHMI, Stanford, USA), the monoclonal mouse-anti-Fz antibody by P. Adler (University of Virginia, USA), the monoclonal mouse-anti-Wg antibody by S. Cohen (EMBL Heidelberg, Germany), and the anti Ci antibody by R. Holmgren (Northwestern University, Chicago, USA). Arm antibodies used were either monoclonal antibody N2-7A1 or polyclonal affinity purified rabbit-anti-Arm N2 (Riggleman et al., 1990). 4D9 mouse anti-En was obtained from the Developmental Study Hybridoma Bank (Iowa, USA).

Fly stocks and genetics

Chromosomal aberrations (Table 1) were obtained from the Bloomington and Bowling Green stock centers unless otherwise indicated. The production of X-chromosomal deletions using *C(1)DX ywf; 1E* and X-chromosomal/Y translocations was described earlier (Wieschaus and Sweeton, 1988; Müller and Wieschaus, 1996). Autosomal compound stocks used were *C(2)v*, *C(3)st e* and *C(4) RM*. The principle crosses employed to generate autosomal deficiencies were described in detail by Merrill et al. (1988). Briefly, deletion embryos were produced by crossing compound females to males bearing chromosomal translocations, transpositions or deficiencies.

The *wg* mutant stock used was wg^{CE7}*/CyO[twi::lacZ]* (Bejsovec and Wieschaus, 1995). *Tp(3;2)N2-27* produces a deletion from 75AB to 80 and a duplication for the same region on the second chromosome. fz^{1} or fz^{R52} was recombined onto *Tp(3;2)N2-27* and the recombinant chromosomes were each maintained in stocks over *In(3L)*fz^{K21}. Both fz^{1} and fz^{R52} alleles produce strong phenotypes in adults. fz^{1} has not been characterized molecularly; fz^{R52} contains a nonsense mutation predicted to encode a protein truncated before the last transmembrane domain (Jones et al., 1996). While both alleles show reduced levels of Fz protein in embryos, fz^{R52} homozygotes shows a more substantial reduction in Fz immunoreactivity (see Fig. 4, unpublished observations).

Phenotypic identification of deficiency embryos

Embryos were collected on yeasted apple juice plates. To analyze the segregation of the translocations in males, we performed test crosses with known embryonic lethal mutations. Males bearing autosomal translocations should produce 1/4 gametes that are deficient for the translocated genomic segment (see Merrill et al., 1988). We used representative point mutations in patterning genes to test the segregation of a given translocation chromosome. In a cross of translocation males to females heterozygous for a given embryonic lethal mutation, 1/8 of the progeny should be mutant for this gene and thus exhibit a characteristic cuticle phenotype. Lethals uncovered by a given translocation are summarized in Table 1. In addition, deletion embryos were identified by observation of living embryos under halocarbon oil (Sigma) and/or by antibody staining against Twist (Twi) and Engrailed (En). For most crosses, the segregation of the deletion class embryos was as expected; in a few cases, the number of deletion embryos was lower than expected.

Table 1. List of chromosomal aberrations used to produce synthetic deficiencies

Name of aberration	Cytology	Phenotype
T(3;1)OR34	3A	N^{-}
T(1;Y)B17	7C	prox. sdt^{-} baz^{-} distal N^{-}
T(1;Y)B139	7F	prox. baz^{-} distal sdt^{-}
T(1;Y)W23	13F	prox. baz^{-} distal sdt^{-}
Tp(2;3)dp[h27]	24F;32B	sph^{-}
T(Y;2)H121	26B	odd^{-}, dpp^{-}, slp^{-}
T(Y;2)J136	26F	odd^{-}, dpp^{-}, slp^{-}, sph^{-}
Tp(2;Y)B231	27D;31E	wg^{-}
Tp(2;3)prd2.27	31B; 33DE	prd^{-}
Tp(2;Y)prd5.12	33A; 35B	prd^{-}
Tp(2;Y)J54	35A; 40	sna^{-}
Tp(Y;2)G	36BC; 40F	fph^{-}
Tp(2;3)P32	41A; 44CD	normal
Tp(2;3)I.707	44B-46DE	en^{-}
Tp(2;Y)G44	44C; 50B	en^{-}, eve^{-}
T(Y;2)H144	47F	en^{-}, Kr^{-}, twi^{-}
T(Y;2)A169	55B	twi^{-}, Kr^{-}
T(Y;2)L116	58A	twi^{-}, Kr^{-}
Tp(2;3)P	58EF; 60DE	twi^{-}
T(Y;3)B234	65B	srw^{-}
Tp(3;Y)B162	65E; 71A	h^{-}
T(Y;3)H167	66F	h^{-}
T(2;3)C309	68F	h^{-}
Tp(3;Y)G63	70A; 77BC	th^{-}
T(Y;3)B223	72AB	h^{-}
T(3;4)A12	73C	*fuzzy cellularization*
Tp(3;Y)J158	73C;79D	kni^{-}
Tp(3;2)N2-27	75AB; 80	kni^{-}
Tp(3;2)FA12	77A; 80F	kni^{-}
Df(3R)ME15 *	81F; 82F	hkb^{-}
Tp(3;Y)G63	83C; 85A	ftz^{-}, zen^{-}, hb^{-}
Tp(3;1)FA11	84DE; 87D	hb^{-}, $neur^{-}$
Tp(3;Y)B216	85F; 91C	htl^{-}
Df(3R)MKRS	87F; 93C	htl^{-}; Dl^{-}
Tp(3;2)S485	89F; 96A	htl^{-}, Dl^{-}, hh^{-}
Tp(3;Y)B219	94C; 100A	tld^{-}, $E(spl)^{-}$, stg^{-}
T(Y;3)P60	99F	*sry* α^{-}, bnk^{-}

The cytological breakpoints significant for the synthetic deficiencies are indicated. In cases where only one breakpoint is shown, the deletion produced reaches from the tip of the chromosome to the cytological position indicated on the table. The column named 'phenotypes' indicates which of the zygotic lethals were tested and are uncovered by the deletion. In the case of *T(1;Y)* s, two deficiency segregants are generated, i.e. proximal and distal to the breakpoint indicated (see Wieschaus and Sweeton, 1988). For reference on chromosomal abberation see Lindsley and Zimm (1992); *information on *Df(3R)ME15* provided by A. T. C. Carpenter (Cambridge, UK).

Immunolabeling

Embryos were dechorionated using commercial bleach and fixed in 4% formaldehyde in PBS (with or without addition of 0.1% Tween 20). Vitelline envelopes were removed by shaking in a 1:1 mixture of methanol and heptane. Embryos were transferred into PBT (PBS, 0.1% Tween 20) and rinsed. After incubation in blocking solution (10% BSA/PBT) for 1 hour at room temperature, embryos were incubated simultaneously or separately with primary antibody combinations diluted in 1% BSA/PBS: rat anti-Wg was used at 1:30, mouse anti-Arm (7A1) at 1:50; mouse anti-Wg at 1:50, rabbit anti-Arm (N2) at 1:100; mouse anti-En (4D9) at 1:10; rabbit anti-Dfz2 at 1:15; mouse anti-Fz (1c11) at 1:5; rat anti-Ci at 1:1. After rinsing in 1% BSA/PBS, embryos were stained with secondary antibodies (goat anti-rat Cy3 1:200, goat anti-rabbit Cy3 1:200, goat anti-mouse Cy3 1:200 (Jackson, USA); goat anti-mouse biotinylated 1:500, goat anti-rabbit biotinylated 1:500 (Vector, USA)). After rinsing in PBT, embryos were incubated in Z-Avidin-FITC (Zymed, USA) (1:200 in PBT) for 40 minutes. Embryos were mounted in either Mowiol containing DABCO (1,4diazabicyclo[2.2.2.]octane) or AquapolyMount (Polysciences). Stained embryos were observed under a confocal microscope (BioRad MRC600 or Leica TSC-NT). Images were processed using Adobe Photoshop on a Power Macintosh.

RESULTS

A genetic screen for zygotic requirements of early Drosophila development

Zygotic requirements for early *Drosophila* embryogenesis can be investigated by analyzing embryos homozygous for deletions of defined genomic regions. A complete coverage of the genome was achieved by Merrill et al. (1988) by using a combination of compound chromosomes and chromosomal translocations to produce synthetic deletions. These synthetic deletions were large and embryos that harbor such deletions displayed severe embryonic abnormalities. Nevertheless, this method was crucial in identifying genes required for the cycle 14 transition, e.g. for the cellularization of the blastoderm (Schejter et al., 1992). To circumvent the deletion of these loci and to allow scoring of later stages, we used additional chromosomal aberrations to produce smaller deletions. The segregation of the translocations in the stocks was tested by non-complementation with known embryonic lethal mutations and is summarized in Table 1 (see Materials and Methods).

According to the published cytology of the chromosomal aberrations, our set of synthetic deletions uncovered a total of 5042 of the 5059 chromosome bands, which relates to 99.7% of the euchromatin. The scheme in Fig 1. indicates the position of 31 overlapping deletions that produce defined embryonic phenotypes. Some genomic regions contain loci required for cellularization (Merrill et al., 1988). Embryos carrying small deletions for *slow phase*, *fast phase*, *serendipity α* or *bottleneck*, while still abnormal during cellularization, allow some analysis of initial *wg* expression and Arm response. The cellularization defects of embryos missing the entire X chromosome were rescued by stable introduction of an autosomal *nullo* transgene into a compound 1 stock (Simpson-Rose and Wieschaus, 1992). Most deletion embryos were analyzed for embryonic phenotypes up to the extended germband stages (stage 9-10). After these stages, some deletion

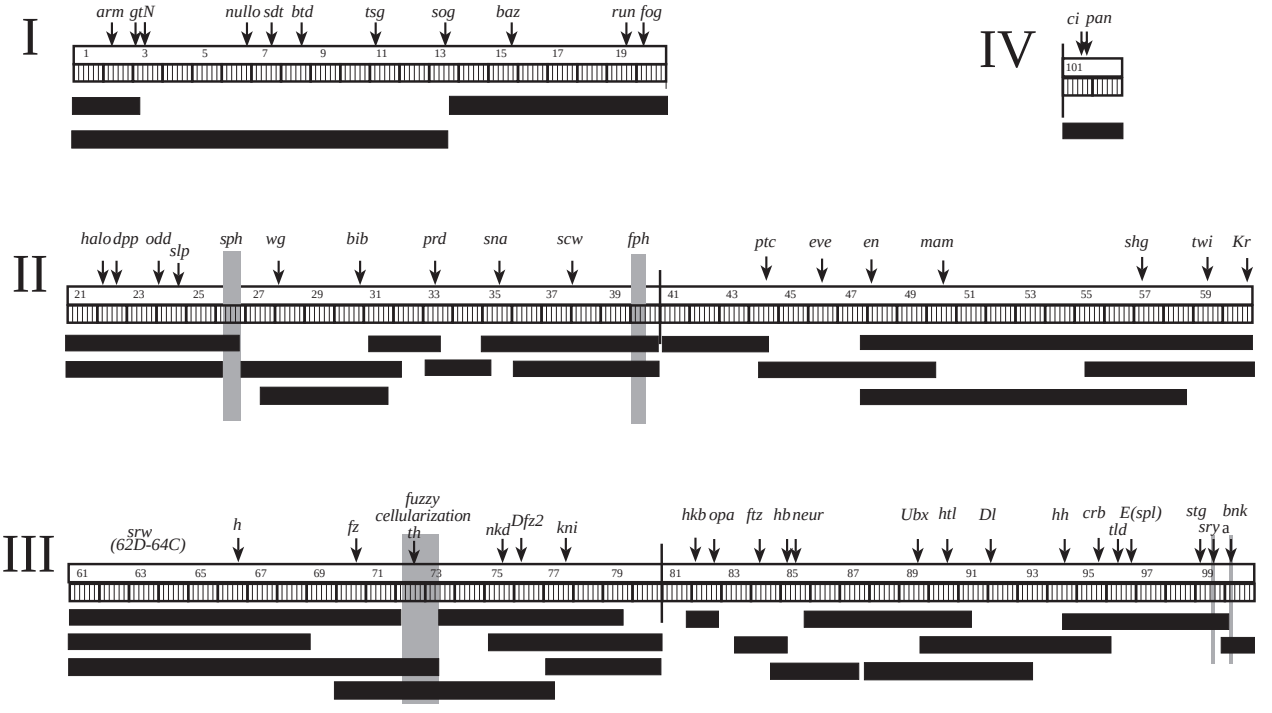

Fig. 1. Coverage of the genome by synthetic deficiencies. The black bars indicate the cytological position of deficiencies produced using compound females crossed to males bearing distinct chromosomal translocations, transpositions and deficiencies (see Table 1 for reference). The vertical bars in gray indicate regions of the genome that when zygotically deleted result in embryos with cellularization defects. The width of the gray bars indicates the preliminary mapping of these regions according to Merrill et al. (1988) and Müller and Wieschaus (unpublished results). The arrows mark the position of a selection of known zygotic lethal mutations that were used as markers for characterization of the deletion embryos. For gene markers see Lindsley and Zimm (1992).

embryos become increasingly abnormal and difficult to analyze. In conclusion, the translocation method provides a tool to rapidly screen for zygotic gene activities required during early developmental processes.

Zygotic control of *wg* expression

To identify genes controlling *wg* expression, we stained embryos derived from the translocation screen with antibodies directed against Wg protein. In wild-type embryos, *wg* is first expressed in the cellular blastoderm and exhibits a dynamic pattern of expression as gastrulation proceeds (van den Heuvel et al., 1989). During germ band extension, the segmental pattern of *wg* expression is established in 15 parasegmental stripes together with a dorsoanterior expression domain (Fig. 2A; van den Heuvel et al., 1989). In general, we find that all deficiencies permit the initial expression of *wg* in the embryo, although the pattern of that expression can be affected by deletion of known segmentation genes (Fig. 2B,C,E). The only deficiencies that do not show expression of *wg* at any developmental stage are those that delete *wg* itself (e.g. *Tp(2;Y)B231*) (Fig. 2D).

Maintenance of *wg* expression at the parasegmental borders depends upon *wg* autoregulation and upon expression of other zygotically active genes, e.g. *ci*, *en*, *gsb*, *hh*, and *slp* (Motzny and Holmgren, 1995; Cadigan et al., 1994; Bejsovec and Wieschaus, 1993; Li and Noll, 1993). As expected, we find that deletion embryos that lack these loci show reduced levels of Wg protein in the ectoderm at later stages (Fig. 3 and data not shown). Embryos lacking the 4th chromosome do not maintain *wg* expression (Fig. 3D). Analysis of candidate 4th chromosomal genes revealed that *ci*, but not zygotic *pan/dTCF*, is required for this maintenance (Fig. 3E).

Most regions show wild-type expression pattern of *wg* or alterations in *wg* expression that can be explained by previously know loci. However, we found that the region 38F to 39F is required for maintenance of *wg* expression. Embryos homozygous for deletions of this region (e.g. *Df(2L)TW65*) show low levels of Wg during gastrulation and completely lose *wg* expression at the extended germ band stage (Figs 2F, 3C). Interestingly, in these deletion class embryos even non-segmental aspects of *wg* expression in the dorsoanterior domain is affected. Although embryos deficient for 38F to 39F do not form a cuticle, the cell morphology at mid-embryonic stages appears to be relatively normal as judged by the cell surface distribution of Arm protein (Fig. 3C). *wg* mRNA expression in embryos homozygous for *Df(2L)TW65* does persist at stages when the Wg protein has faded (Müller and Wieschaus, unpublished results). Together these results suggest that the loss of *wg* protein expression might be a post-transcriptional defect and is not due to general degeneration of embryos homozygous for *Df(2L)TW65*. Another genomic region that affects aspects of *wg* expression is the genomic region 70D to 71A. In embryos with large synthetic deficiencies including 70D to 71A, *wg* expression is more reduced than in smaller deficiencies that do not extend into this region (Fig. 3G,H). In summary, our results suggest that regulation of *wg* expression might only require zygotic expression of relatively few as yet unidentified genes.

Zygotic requirements for Wg signal transduction

In normal embryos, Arm protein accumulates in regions that flank the *wg* stripes and other *wg*-expressing domains (Peifer et al., 1994) (Figs 2, 3). We asked whether this response is impaired in the various deletion embryos generated in our translocation screen. All deletion embryos expressing *wg* show at least some Arm striping response; the only embryos that do not show Arm stripes are those in which the *wg* gene had been deleted (Figs 2, 3). Even very large deficiencies (e.g., those representing the entire right arm of the second chromosome, or most of the X chromosome) showed elevated levels of

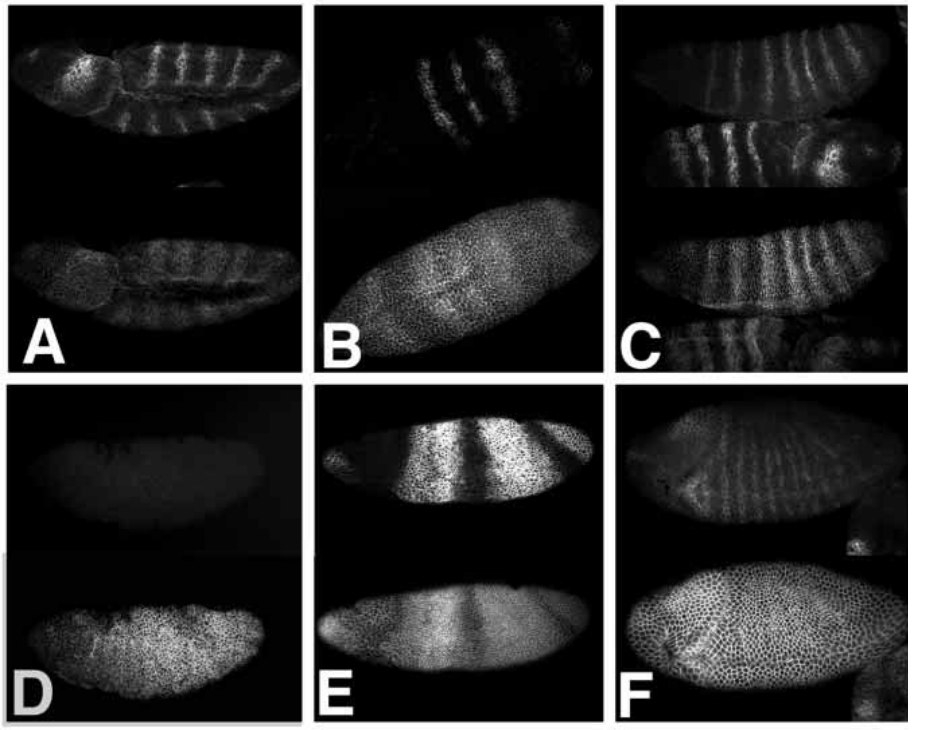

Fig. 2. Expression of Wg and Arm in gastrula stage embryos. Confocal images of double labeled embryos are shown. The upper embryo in each panel represents the anti-Wg staining; the lower panel shows the corresponding anti-Arm staining. (A) Wild-type pattern of Wg and Arm in extended germband embryo (stage 9). Note the segmentally repeated, elevated levels of Arm staining (Arm stripes). (B) The ventral view of a *nullo X;1E* embryo is shown. In *nullo X* embryos only four *wg* stripes are generated due to absence of the segmentation genes *gt* and *run*. (C) Embryo deleted for 21A-26B using *T(Y;2) H121*. Only half the number of *wg* stripes is seen, because *slp* and *odd* are deleted. (D) Embryo deficient for *wg* from the cross *C(2)v* × *Tp(2;Y)B231* (deleted for 27D-31E). (E) *nullo 2R* embryos show strongly reduced number of stripes due to absence of *Kr* and *eve*. In addition, *wg* stripes are expanded when compared to wild-type embryos, due to deletion of the segment polarity gene *ptc*. (F) Embryo homozygous for *Df(2L)TW65* (38A-39EF) at an early gastrula stage (stage 8).

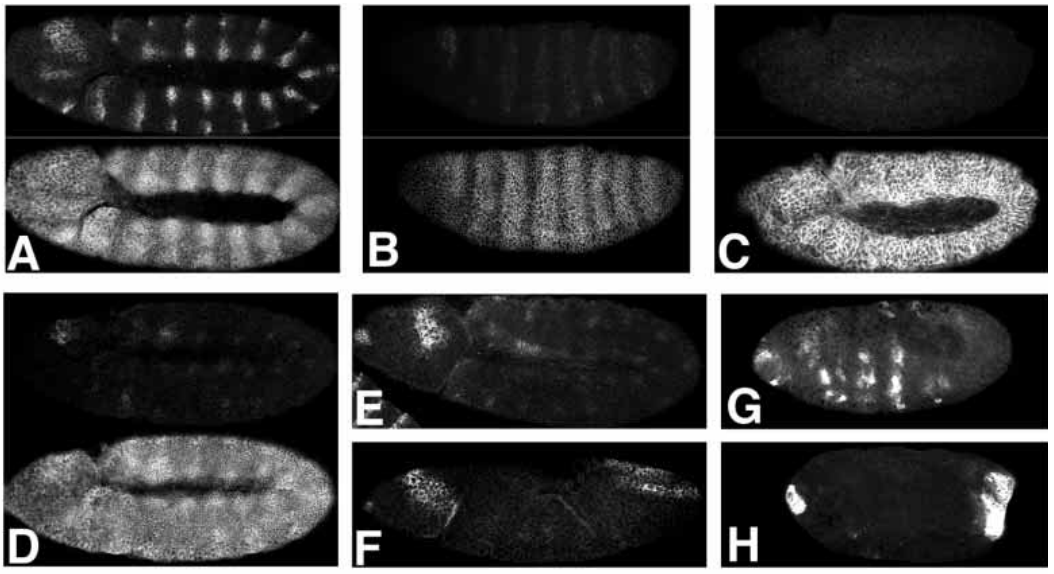

Fig. 3. Maintenance of *wg* expression in postgastrula stages embryos (stages 10 and 11). Double labeled embryos in A-D are: Wg staining upper part, Arm staining lower part of the panel. E-H show single labeling with anti-Wg. (A) Wild-type expression pattern of Wg and Arm in an extended germband embryo (stage 10). (B) Reduction of Wg levels in an embryo deleted for *slp* (cross: *(C(2)v × T(Y;2)H121*). *wg* expression is still present in the head but is not in the focal plane of this image. (C) Postgastrula embryos homozygous for *Df(2L)TW65* do not maintain *wg* expression. Note that the anterior expression domain is absent. (D) *nullo 4* embryo shows reduced levels of Wg. (E) Embryos homozygously mutant for *ci* (ci^{ce2}) show a reduction of Wg staining similar to *nullo 4* embryos. (F) Expression of *wg* in deletion embryo lacking *en* (from the cross *C(2)v × Tp(2;3)I.707;* deleted for 46E-49E). Head and posterior expression of *wg* is maintained. (G) Embryo deleted for 61A to 68E (cross: *C(3)se × T(2;3)C309*). The number of Wg stripes is reduced, because the deletion uncovers *h*. (H) A stronger reduction of Wg stripes is seen in embryos deleted for 65E-71A (cross: *C(3)se × Tp(3;Y)B162*).

cytoplasmic Arm in cells surrounding *wg* expressing domains. Response to Wg signal does not appear to require an intact epithelium or the presence of normal adhesive junctions. We have previously shown that, in embryos lacking the X-linked genes *stardust* (*sdt*) and *bazooka* (*baz*), cells fail to form a normal zonula adherens and lose their epithelial morphology at the onset of gastrulation (Müller and Wieschaus, 1996). In spite of these defects, cells lacking *sdt* and *baz* and almost the entire X-chromosome still show Arm accumulation in response to Wg (Fig. 2B).

Although two *Drosophila* frizzled family members (Dfz2 and Frizzled) have been shown to function as Wg receptors in cell culture assays (Nusse et al., 1997), their roles in embryonic signaling have not been characterized. We detect both receptors in ectodermal derivatives during midembryonic stages (Fig. 4). As previously reported (Bhanot et al., 1996), Dfz2 is initially expressed in a broad domain but sharpens to a segmentally repeated pattern by stage 10. Highest levels of Dfz2 are detected in cells immediately posterior to the En stripe (Fig. 4A-C). This expression grades off posteriorly and may not extend to the Wingless-expressing domain. Although Fz initially appears to be uniformly distributed during gastrulation, it too resolves into a periodic pattern by stage 9 (Fig. 4D,E). In their final form, stripes of high Fz expression are broader than those observed with Dfz2 and appear to encompass the entire cellular domain between adjacent En stripes. The Fz expression domain corresponds to the cells expressing Ci (Fig. 4G-I), and overlaps at its posterior region with the row of cells expressing Wg (Fig. 4J-L). We conclude that Dfz2 and Fz are expressed in overlapping domains during gastrulation and show more restricted expression patterns later in development. By stage 10, both receptors show strongly reduced expression in cells that express En.

Homozygotes for *fz* are viable and fertile, although they show disrupted bristle and hair patterns as adults. *fz* embryos derived from homozygous *fz* mothers show normal Arm response to Wg signaling (Fig. 4M). To delete *Dfz2*, we crossed transpositions (*Tp(3;Y)J158* or *Tp(3;2)N2-27*) affecting the proximal region of 3R to compound-3 females (Fig. 1; Table 1). *Dfz2*-deficient embryos derived from this cross can be recognized because they also lack a wild-type *knirps (kni)* gene with corresponding defects in abdominal segmentation. Although the *wg* expression pattern in such embryos is altered, Arm protein still accumulates in regions where *wg* is expressed (Fig. 4N). To test the possibility that *fz* and *Dfz2* function redundantly, we constructed recombinant chromosomes carrying *Tp(3;2)N2-27* and different *fz* alleles (fz^{1} or fz^{R52}). Embryos derived from compound-3 mothers mated to males carrying these chromosomes still show Arm accumulation in response to Wg (Fig. 4O). Since the compound-3 mothers are wild type with respect to *fz* and *Dfz2*, a maternal contribution from either gene might mask potential zygotic phenotypes. To eliminate maternal *fz*, we introduced the *fz Tp(3;2)N2-27* chromosome into a *fz* mutant background (fz^{1} *Tp(3;2)N2-27* /*In(3L)*fz^{K21} or fz^{R52} *Tp(3;2)N2-27* /*In(3L)*fz^{R52}). The phenotypically *kni* embryos from these stocks lack maternal and zygotic *fz* activity and are zygotically mutant for *Dfz2*. Such embryos no longer show high levels of Arm protein in response to Wg signaling (Fig. 4P,Q). The inability of *fz Dfz2* mutant cells to respond to Wg signal requires removal of both maternal and zygotic *fz*. When *fz Tp(3;2)N2-27* /*In(3L)*fz^{K21} are crossed to *Tp(3,Y)J158* males, the resultant embryos lack *fz* maternally and *Dfz2* zygotically, but show normal Arm stripes (Fig. 4R). Since *fz* mutant embryos from *fz* homozygous mothers respond to Wg signaling, the failure of mutant embryos in the *fz Tp(3;2)N2-27* /*In(3L)*fz^{K21}stock to accumulate Arm stripes is most likely due to loss of zygotic *Dfz2*. Maternal expression of *Dfz2* is not sufficient for Wg signaling, because females in the above cross carry both copies of the *Dfz2* gene.

Does the *fz Dfz2* double mutant block all Wg signaling?

In the ectoderm, Wg acts to maintain its own expression and expression of *en* in adjacent cells (Heemskerk et al., 1991; Bejsovec and Martinez-Arias, 1991). Removal of *fz* and *Dfz2* affects visible cytoplasmic accumulation of Arm, reflecting a common role for the two genes at stages when their expression overlaps. If elimination of the two genes is sufficient to block all Wg responses, *wg* and *en* expression should also not be maintained. We find that Wg expression in embryos deficient for *fz* and *Dfz2* is lost in a temporal pattern similar to that seen in *wg* mutants (Fig. 5A-D). In contrast, the effect on *en* expression appears more moderate. Expression of *en* is reduced in *fz Dfz2* double mutant embryos when compared to wild type (Fig. 5E,H), but persists longer than in *wg* mutant embryos (Fig. 5F,H). Since the *Tp(3;2)N2-27* deficiency also uncovers *naked* (*nkd*), *en* stripes are expanded in the deletion embryos (Fig. 5G). This expansion of *en* stripes in the absence of *nkd* depends on *wg* (Bejsovec and Wieschaus, 1993) and is blocked in embryos doubly mutant for *fz* and *Dfz2* (Fig. 5G,H).

The partial maintenance of *en* expression suggests that the *fz Dfz2* double mutant does not completely eliminate all Wg reception. Our immunostaining of postgastrula embryos reveal only low levels of these two receptors in *en* expressing cells; it is possible that some other frizzled-type receptor contributes to Wg-mediated En maintenance. Given the relatively high staining levels for Dfz2 and Fz in the Ci domain, the effect of the double mutant on Wg expression, the two genes may be the principle receptors involved in Wg maintenance.

Internalization of Wg protein occurs in absence of Wg signal transduction

Upon binding to the cell surface, Wg protein is internalized by the receiving cell (van den Heuvel et al., 1989; Gonzalez et al., 1991; Bejsovec and Wieschaus, 1995). Internalization of Wg is evident from immunostainings of wild-type embryos, where Wg protein can be detected in dots outside the *wg* expressing cells (Fig. 6A). These dots presumably represent cytoplasmic vesicles indicating intermediates of the endocytic pathway (Gonzalez et al., 1991). When endocytosis is blocked using the temperature-sensitive *shibire* mutation, the receiving cells do not internalize Wg protein and the expressing cells accumulate

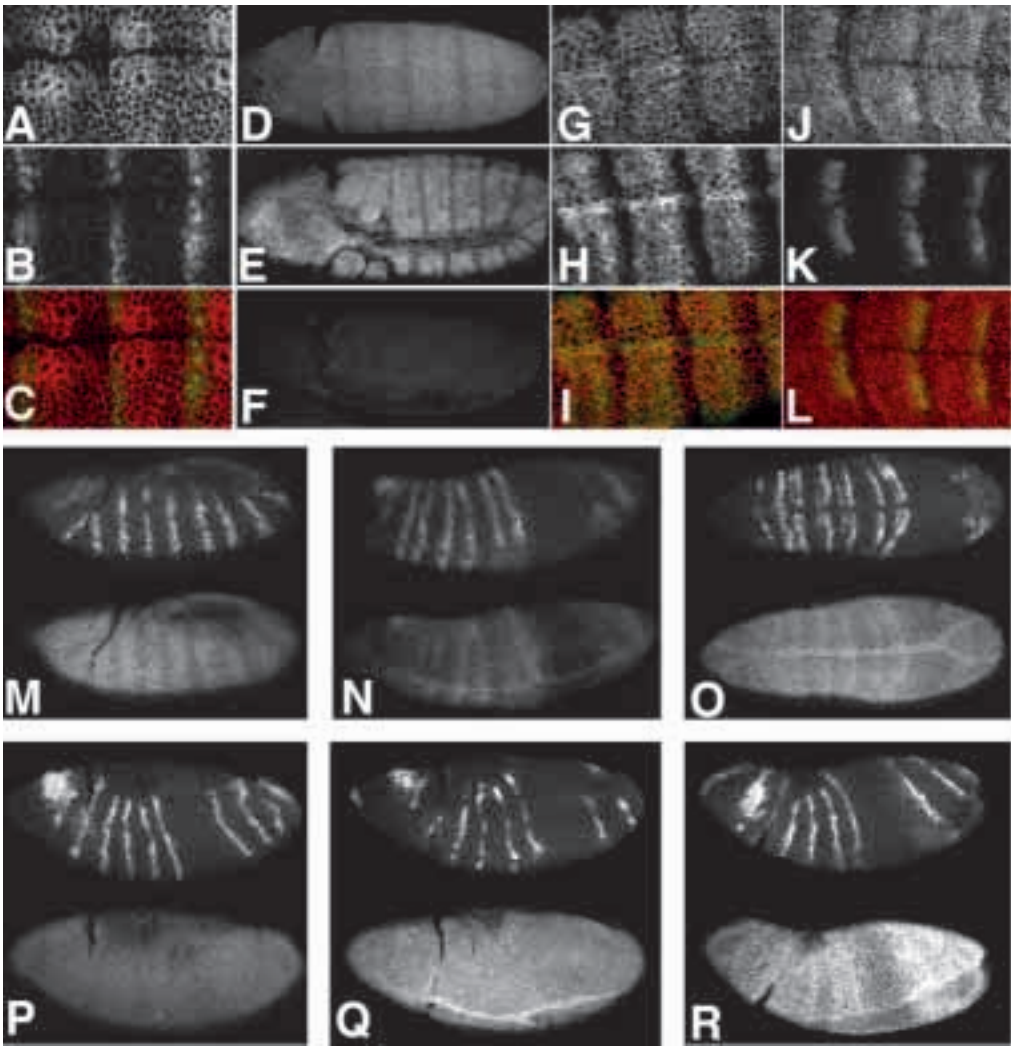

Fig. 4. Immunolocalization of Dfz2 and Fz and the Arm response in embryos mutant for *fz* and *Dfz2*. To localize Dfz2 protein, wild-type embryos (stage 10) were double labeled with antibodies against Dfz2 (A) and antibodies against En (B); overlay of both signals is shown in C. Fz protein localization is shown in wild-type embryos at stage 9 (D) and stage 11 (E). (F) Fz staining is strongly reduced in an embryo mutant for *fz* derived from homozygous fz^{R52} mutant mothers. To elucidate the position of the segmentally repeated Fz stripes, embryos were double labeled with antibodies against Fz (G) and Ci (H); I shows the merged image. Alignment of Fz stripes with Wg is shown in J (anti Fz), K (anti Wg), and L (merged image). (M-R) Arm response in *fz Dfz2* mutant embryos was analyzed by double labeling with antibodies against Wg (upper panels) and Arm (lower panels). (M) *fz* mutant embryos derived from *fz* mutant mothers show normal Arm striping (from stock fz^{1} *Tp(3;2)N2-27/In(3L)fz* ^{K21}). (N) Deletion embryos from the cross: *C(3)se* × *Tp(3;Y)J158* are zygotically deficient for *Dfz2* and show normal Arm response. Note abdominal segmentation defect based on the absence of *kni*, which is also uncovered by the synthetic deficiency. (O) Deletion embryo derived from *C(3)se* × fz^{1} *Tp(3;2)N2-27/In(3L)fz* ^{K21}; these embryos are zygotically deficient for *fz* and *Dfz2* and still show normal Arm response. Arm response is strongly reduced in two distinct *fz Dfz2* double mutant combinations derived from fz^{1} *Tp(3;2)N2-27/In(3L)fz* ^{K21} (P) or from fz^{R52} *Tp(3;2)N2-27/In(3L)fz* ^{K21} (Q). (R) Normal Arm response is seen in deletion embryos derived from the cross fz^{1} *Tp(3;2)N2-27/In(3L)fz* ^{K21} × fz^{+}, *Tp(3;Y)J158*, which are maternally mutant for *fz* and lack *Dfz2* zygotically.

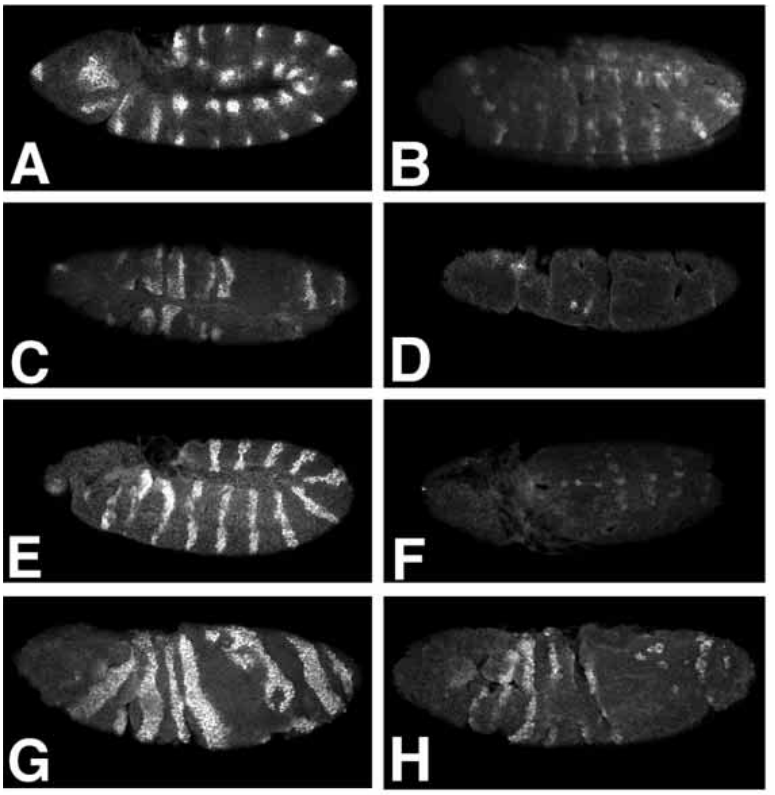

Fig. 5. Maintenance of *wg* and *en* expression in *fz Dfz2* mutant embryos. Embryos were collected for 1 hour, aged for 7 hours at 25°C, fixed, and immunolabeled for either Wg (A-D) or En (E-H). (A,E) Wild-type expression of Wg and En, respectively. (B,F) wg^{CE7} mutant embryos: while Wg protein is still present at low levels (B), En protein in the ectoderm is at the limit of detection (F). (C,G) Embryos deleted for *Dfz2* (*C(3)se* × *Tp(2;3)N2-27*). Note expansion of *wg* and *en* expression domains. (D,H) *fz Dfz2* double mutant embryos (from stock fz^{R52} *Tp(3;2)N2-27/In(3L)fz* ^{K21}). Wg protein has faded in the double mutant at this time of development except for some expression in the dorsoanterior domain (D). (G) En protein is reduced, though clearly detectable, but does not show the expansion of the stripes as seen in the *Dfz2* single mutant (compare G with H).

high levels of Wg protein (Fig. 6B; see also Bejsovec and Wieschaus, 1995). Altogether these results suggest that Wg is internalized by receptor-mediated endocytosis. Surprisingly, in the *fz Dfz2* double mutant, Wg protein still shows a vesicular staining pattern in cells outside the *wg* expressing domain (Fig. 6C). This result indicates that internalization of Wg does occur in cells where the transduction of the Wg signal to the cell interior is strongly reduced.

If the frizzled receptors are not essential for internalization of Wg protein, there might be other cell surface molecules to fulfill this function. In our screen, we have identified one genomic region that shows an unusual Wg protein distribution. In embryos homozygously deficient for the region 36B to 40F, Wg protein can be found predominantly in single rows of cells and is rarely found in cells adjacent to the *wg* expressing cells (Fig. 6D). Thus, this genomic interval may contain a gene or genes involved in transport of Wg protein.

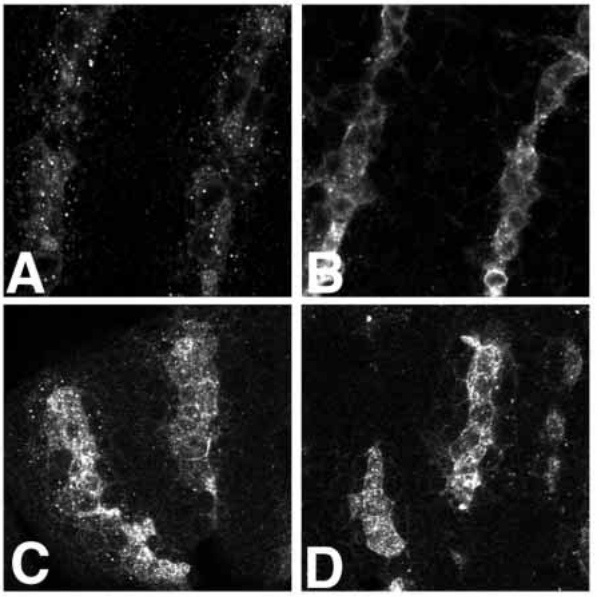

Fig. 6. Embryos mutant for *fz Dfz2* exhibit a normal vesicular Wg distribution. Wild-type embryos (Oregon R; A) and shi^{ts} embryos (B) were collected for 5 hours and then shifted to 32°C, cultured for 1.5 hours and fixed (stage 10). Note that in the wild type, Wg protein shows a vesicular distribution in the interstripe region (A). In a *shi* mutant embryo, the vesicular staining of Wg is absent (B). (C) *fz Dfz2* double mutant embryo from fz^{1} *Tp(3;2)N2-27/In(3L)fz* ^{K21} stock at stage 9. Note vesicular distribution of Wg in the interstripe region. (D) Embryo at similar developmental stage deficient for the genomic segment 36 to 40 (cross *C(2)v* × *Tp(2;Y)G*) shows reduced vesicular staining in the interstripe region. Non-deletion, sibling embryos from the latter cross produced normal Wg protein distribution at corresponding developmental stages (data not shown).

DISCUSSION

Translocation screen: a rapid method to analyze zygotic requirements for early embryogenesis

The translocation screen covers more than 99% of the genome using a total of 31 crosses. For comparison, conventional deficiency screens are performed with about 161 stocks to obtain a coverage of about 70% (Harbecke and Lengyel, 1995). Since females of conventional deletion stocks are themselves heterozygous for the genes uncovered by the deficiency, the phenotype of their embryos may also reflect maternal dosage rather than deletion of zygotically active genes. In contrast, the compound females used in our screen supply the embryos with wild-type maternal dosage. Thus the phenotype of a given

deletion class embryo using compound females strictly reflects the zygotic genotype uncovered by a particular deficiency segregant.

The translocation screen generally produces embryos that can be analyzed well into gastrulation. One of the limitations of the screen is that after germ band extension, most aneuploid embryos develop very abnormally. For this reason, the analysis of the maintenance of *wg* expression was difficult. We assume that the defects observed in late-stage deficiency embryos represent additive effects arising from simultaneous deletion of many genes and effects resulting from haploidy for a given chromosome arm. To minimize such problems, we sometimes found it necessary to repeat the analysis of complicated regions using conventional deficiencies (a complete list of the stocks used in this study can be obtained from http://www.uni-duesseldorf.de/WWW/MathNat/Genetik/muellear.html).
Within these limits, the translocation screen represents the first method to comprehensively search for zygotically active genes that are required for early embryogenesis.

Zygotic control of *wingless* expression

The expression of *wg* is influenced by a complex set of zygotically active genes. These gene activities regulate specific aspects of the segmental pattern of *wg* expression in the embryo and loss-of-function mutations produce alterations in the pattern of the *wg* stripes. We observed corresponding changes in *wg* expression in the deficiency embryos, all of which can be explained by deletion of previously characterized genes. Although a large number of genes affect the pattern of *wg* expression, no single genomic region other than *wg* itself is absolutely required for production of Wg protein. These results suggest a model in which *wg* would in principle be expressed by all cells in the ventral ectoderm, if its expression were not regulated by the combinatorial action of patterning genes. This model would predict that, after removal of zygotic pattern constraints, *wg* should be expressed throughout the segment. In fact, *wg* is expressed uniformly in the ventral ectoderm in embryos triply mutant for *ptc*, *nkd* and *en* (Bejsovec and Wieschaus, 1993).

In contrast to the requirements for initial *wg* expression, maintenance of its expression depends on the activity of other segment polarity genes and on *wg* itself (Bejsovec and Martinez Arias, 1991; Ingham and Hidalgo, 1993). Some of the genes that we found to be required for persistent expression of *wg*, like *hh*, *en*, *gsb*, *ci* and *slp*, have been implicated in the regulation of *wg* expression before (Ingham and Hidalgo, 1993; Bejsovec and Wieschaus, 1993; Li and Noll, 1993; Cadigan et al., 1994; Motzny and Holmgren, 1995). That *pan/dTCF* is dispensable for *wg* maintenance could be explained if maternal gene product is supplied in sufficient quantities to compensate for lack of zygotic expression (van de Wetering et al., 1997). In addition to known zygotic regulators of *wg* expression, we found that the genomic interval 38F – 39F is required for all aspects of maintenance of *wg* expression; *wg* expression not only fades in the parasegments, but also in the head and the posterior terminal region.

Redundancy of *fz* and *Dfz2* in Wg signaling

The frizzled transmembrane proteins constitute a large family of structurally closely related molecules. The structural conservation between different members of the frizzled family is most evident in a conserved cysteine-rich domain on the extracellular portion of the molecule, which also constitutes the putative ligand-binding domain (Wang et al., 1996). The two frizzled genes analyzed here, *fz* and *Dfz2*, can both bind Wg in cell culture assays. In both cases, this binding leads to accumulation of cytoplasmic Arm levels (Nusse et al., 1997). The similarity of their response to Wg, as well as their similar structures, may provide the functional basis for the redundancy that we observe in our experiments. This redundancy provides the first genetic evidence that Fz, the founding member of the frizzled receptor family, utilizes Wg as one of its natural ligands during normal development. In this view, the tissue polarity phenotype observed in mutant *fz* adults would reflect only a subset of the gene's roles during development. The tissue polarity phenotype differs from Wg pathway defects in the embryo in that it does not involve changes in cell fate and may not even involve changes in gene expression. In the simplest models, bristle polarity requires that cells remember the surface that receives the external polarizing signal and utilize the resultant surface differences to orient cytoskeletal outgrowth. Although our results argue that Fz can respond to secreted Wg, they do not necessarily indicate that Wg functions as the polarizing signal during Fz's role in controlling tissue polarity. Such a role might require a more specialized ligand, or cofactors that would supply the necessary spatial stability of the ligand complex on the surface of the responding cell. It is possible, however, that the role of Fz in embryonic Wg signaling may also involve some aspects analogous to the gene's role in tissue polarity, given the polarized pattern of Wg accumulation in the epidermis during midstages of embryonic development (Gonzalez et al., 1991).

The apparent redundancy of the two frizzled family members with respect to stabilization of cytoplasmic Arm protein does not preclude distinct functions for the two receptors during embryogenesis. Experiments based solely on Arm accumulation are necessarily somewhat crude and may not detect subtle differences in the function of the two genes. Earlier studies have shown that Wg signaling is required in discrete temporal phases and for at least two different aspects of epidermal patterning: cell diversity and specification of cells secreting naked cuticle. *wg* alleles exist that affect only a subset of its role epidermal patterning (Bejsovec and Wieschaus, 1995). This complexity may reflect differential utilization of the two frizzled receptors, their differing sensitivities to concentration of Wg ligand, or different requirements for cofactors or cell surface glucosaminoglycans (Häcker et al., 1997). The large size of the *Tp(3;2)N2-27* deficiency precludes a detailed analysis of final differentiation phenotypes of *Dfz2* mutants. Such an analysis, however, may be possible once point mutations are available that specifically eliminate this receptor.

When we analyzed two known downstream responses of Arm signaling, maintenance of *en* and *wg* expression, we found that these responses were affected differentially. One way to obtain distinct responses of combinations of redundantly acting receptors is their differential expression. Although the two receptors that we studied initially show relatively uniform distributions in the epidermis, they ultimately come to be expressed in distinct patterns. The different spatial distributions may reflect different roles in development: Fz is more generally

distributed in the entire anterior compartment of each segment, Dfz2 predominantly in the regions that normally give rise to the denticle belt far from the Wg source. At late stages, both receptors are only detected at low levels in the *en* expressing cells. The impaired En maintenance observed in the *fz Dfz2* double mutant argues that the two receptors have a function at least at some stage in these cells. However, since the double mutant phenotype does not eliminate all *en* maintenance, removal of Fz and Dfz2 does not totally eliminate the ability of *en* expressing cells to respond to Wg signal. This observation raises the possibility that *en* maintenance at late stages of development may involve other receptors capable of responding to Wg signals.

Internalization of Wg requires factors other than known frizzled receptors

Evidence for separate mechanisms of Wg transport and Wg signal transduction comes from the observation that *wg* can be mutated differentially (Besjovec and Wieschaus, 1995). Embryos mutant for wg^{PE2} are defective in secretion of naked cuticle, whereas wg^{PE4} mutants secrete naked cuticle, but are defective in generating denticle diversity. Hays et al. (1997) presented evidence that the Wg^{PE4} mutant protein is specifically defective in intercellular transport. These results suggested that distinct domains of Wg might bind to different proteins, which in turn might be either involved in transport or in transduction of the signal. The distribution of Wg in *fz Dfz2* mutant embryos provides further support for this model. Although Wg signaling is strongly reduced in the double mutant, we did not observe a corresponding decrease in vesicular localization of Wg (Fig. 6). Thus our double mutant combination affects Arm response without a corresponding effect on Wg's ability to bind to the surface of neighboring cells or be internalized. The *fz* mutant alleles used here, although genetically null, may allow normal ligand binding without signaling. It is also possible that as yet undetected maternal contributions of *Dfz2* may account for the internalization. We favor however a third possibility, namely that binding utilizes proteins different than the frizzled receptors that mediate signaling.

The failure of *shi* mutants to internalize Wg protein suggests that receptor-mediated endocytosis is the mechanism by which Wg is incorporated into cells. When endocytosis is blocked, cells that bind Wg still show typical Arm striping (Bejsovec and Wieschaus, 1995) suggesting that internalization is not essential for receptor activation. These observations provide a partial complement to the normal internalization that we observe in the *fz Dfz2* double mutant. The two observations together suggest that Wg binding and signal transduction may be genetically separable. Other components involved in Wg presentation and internalization are thus far elusive. Our translocation screen identified a single genomic region that might contain a candidate gene involved in Wg protein transport. Embryos deficient for this region show a strong reduction of vesicular Wg protein localization. This phenotype is not identical to that produced by *shi* and may therefore provide a handle on processes more specific to the Wg ligand, presentation, or transport.

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