**wingless** signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo

Mark Peifer¹*, Dari Sweeton², Michael Casey¹ and Eric Wieschaus²

¹Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280, USA
²Department of Molecular Biology, Princeton University, Princeton, NJ 08544-1014, USA

*Author for correspondence

**SUMMARY**

wingless/wnt-1 signaling directs cell fate during development. Genetic analysis in *Drosophila* identified genes that may encode components of the wingless signal transduction system. *Drosophila* Armadillo, homolog of vertebrate β-catenin, is required for wingless signaling. Unlike armadillo RNA, Armadillo protein accumulates non-uniformly in different cells of each embryonic segment. We found that cells alter their intracellular distribution of Armadillo in response to Wingless signal, accumulating increased levels of cytoplasmic Armadillo relative to those of membrane-associated protein. Levels of cytoplasmic Armadillo are also regulated by Zeste-White 3 kinase. Analysis of double mutants demonstrates that Armadillo's role in wingless signaling is direct, and that Armadillo functions downstream of both wingless and zeste-white 3. We present a model for the role of Armadillo stripes in transduction of wingless signal.

Key words: wingless, Zeste-white 3 kinase, Armadillo, β-catenin, signal transduction, *Drosophila*

**INTRODUCTION**

In insects and vertebrates, cell-cell signaling molecules of the wingless/wnt family control cell fate decisions (reviewed in Peifer and Bejsovec, 1992; McMahon, 1992). *Drosophila* wingless (wg) is critical for development of many tissues, including the embryonic cuticle (Nüsslein-Volhard and Wieschaus, 1980). Embryonic epidermal cells choose different fates dependent on their position within each segment. One reflection of these decisions is that anterior cells secrete denticles while posterior cells secrete naked cuticle. wg mutations disrupt this pattern.

*wg* encodes a secreted protein (van den Heuvel et al., 1989) that is the *Drosophila* homolog of vertebrate *wnt-1*. wg RNA is expressed by a subset of the cells of each segment; Wingless is secreted by these cells and assumes a graded distribution, at least early in embryogenesis (Gonzalez et al., 1991). wg acts as a cell-cell signal (reviewed in Peifer and Bejsovec, 1992). In cooperation with other segment polarity gene products, wg promotes cell fate diversity along the anterior-posterior axis (Bejsovec and Wieschaus, 1993), but it remains unclear whether wg acts as a graded morphogen or transmits a different signal. Other segment polarity genes sharing the *wg* phenotype may be required for secretion, reception and/or interpretation of *wg*. Our focus is the *armadillo* gene (*arm* = gene, Armadillo = protein). *arm* is required for reception and/or interpretation of *wg* signal (Wieschaus and Riggleman, 1987; Peifer et al., 1991). Other proteins, such as the Ser/Thr protein kinase *zeste-white 3* (zw3, also known as *shaggy*) are also required for this process (Siegfried et al., 1990; Bourouis et al., 1990; Siegfried et al., 1992).

Armadillo, homolog of the vertebrate adhesive junction proteins β-catenin and plakoglobin (Peifer and Wieschaus, 1990; McCrea et al., 1991), is part of a membrane-associated multiprotein complex similar to the vertebrate adherens junction. This complex includes Armadillo, a glycoprotein likely to be a cadherin homolog (by analogy to vertebrate adherens junctions), and the *Drosophila* α-catenin homolog (Peifer, 1993; Oda et al., 1993). Armadillo and adherens junctions are required to maintain cell adhesion and actin cytoskeletal integrity (Peifer et al., 1993). The connection between adherens junctions and *wg* signaling was unexpected. This connection has two possible explanations. One is indirect. Many cell-cell signaling molecules are localized to adherens junctions (e.g. Tsukita et al., 1991). If the putative wg receptor is localized there, abnormalities in the adherens junction of *arm* mutants might cause the wg receptor to be mis-localized or non-functional. Alternately, *arm* could play a direct role in reception of or response to *wg* signal.

One clue to Armadillo function is the finding that Armadillo accumulates differently in different cells within each segment. Although *arm* RNA is uniformly distributed (Riggleman et al., 1989), Armadillo protein distribution is segmentally striped (Riggleman et al., 1990). These are not ON/OFF stripes; all cells accumulate Armadillo, but, as we show here, Armadillo intracellular distribution and levels of Armadillo accumulation are altered in a segmentally repeated fashion. Armadillo stripes coincide roughly with the graded Wingless protein stripes.
Our interest is in cellular mechanisms that generate Armadillo stripes and the role of these stripes in wg signal transduction. We have found that Armadillo stripes result from both effects on the amount of Armadillo in 'stripe cells' and dramatic alterations in its intracellular localization in those cells. In the absence of wg signal, most cellular Armadillo is part of a membrane-associated complex (Peifer, 1993). Here we show that, in response to wg, cells accumulate much higher levels of cytoplasmic Armadillo. This change is correlated with the fates that cells adopt. Mutations in wg and zw3 have opposite effects on epidermal cell fate. Each mutation affects both cell survival and cell fate. All surviving cells in a wg mutant secrete denticles, developing like the wild-type cells in the anterior of each segment that do not receive wg signal. Conversely, all surviving cells in a zw3 mutant secrete naked cuticle, like posterior wild-type cells receiving maximal wg signal. We show that wg and zw3 mutations also have opposite effects on Armadillo stripes. In wg mutant embryos, all cells have primarily membrane-associated Armadillo-like wild-type anterior cells, while all cells in a zw3 mutant accumulate high levels of cytoplasmic Armadillo-like wild-type posterior cells. We used genetic epistasis to show that arm lies downstream of zw3 in the wg signaling pathway, consistent with our observation that zw3 is required for Armadillo phosphorylation (Peifer et al., unpublished data). We end by proposing a direct role for Armadillo in wg signal transduction.

MATERIALS AND METHODS

Fly stocks and production of germ-line clones

arm mutants are described in Peifer and Wieschaus (1990). Alleles used were zw3M11-1 (Siegfried et al., 1992) and wgK22 (Nüsslein-Volhard et al., 1984). Identification of wg mutations was done using a recombinant chromosome carrying a twist mutation. arm and zw3 mutant embryos were identified using a recombinant chromosome carrying twisted gastrulation. Germ-line clones were produced using the yeast recombinase-based FLP-DTS system (Chou and Perrimon, 1992). Briefly, Fs(1)ovoD1 FRT101/Y; hs-flp-F38 males were crossed to arm and/or zw3 FRT101/FM7 females. FRT101 is an insertion of the FLP site-specific recombination site near the base of the X chromosome, proximal to arm, zw3 and the dominant female-sterile, Fs(1)ovoD1, hs-flp-F38 is an autosomal insertion of heat-shock-promoter-driven FLP recombinase. Recombinase was induced by heat shocking second-third instar larvae at 37°C for 3-5 hours, leading to site-specific mitotic recombination between FRT101 sites on homologous chromosomes in a subset of cells in animals of the genotype Fs(1)ovoD1 FRT101/arm and/or zw3 FRT101, and producing clones either homozygous for arm and/or zw3 or for Fs(1)ovoD1. Adult progeny of the genotype Fs(1)ovoD1 FRT101/arm and/or zw3 FRT101 were crossed to wild-type males and eggs collected. Because of the dominant female-sterile mutation only flies with germ-line clones homozygous for arm and/or zw3 develop ovaries and lay eggs.

Antibodies, immunocytochemistry, immunoblotting and membrane fractionation

Most experiments were done with the polyclonal N2 anti-Armadillo antibody (Riggleman et al., 1990) or the monoclonal antibody, N2-7A1, directed against the same region of Armadillo (amino acids 67-123). These two antibodies give indistinguishable results. The C-terminal antibody C3-4H10 is a monoclonal antibody directed against a fusion protein containing the C-terminal 43 amino acids of Armadillo. Armadillo stripes can also be detected with an antibody against a different region of the N-terminal domain of Armadillo (amino acids 21-68), N1-7C7. The procedure for immunofluorescence is as described in Peifer et al. (1992), except where noted when heat/methanol fixation was used (Miller et al., 1989; Peifer, 1993). This latter fixation procedure appears to allow loosely bound Armadillo to wash away, preserving only the most tightly bound protein. Immunoblotting was done as in Peifer et al. (1992), using monoclonal anti-Armadillo antibody 7A1. The antibody used for controls was monoclonal anti-Bicaudal D (Suter and Steward, 1991), diluted 1:100. Blots previously reacted with anti-Armadillo antibody were treated as recommended by Amersham to remove that antibody, and reprobed with anti-BicD antibody. Membrane fractionation was as in Peifer (1993).

RESULTS

Morphological characterization of the segmental stripes of Armadillo

We extended the previous description of Armadillo stripes (Riggleman et al., 1990) by using confocal microscopy. Armadillo stripes arise soon after the onset of gastrulation and reach maximum intensity by embryonic stage 9 (Figs 1, 2B, Wieschaus and Nüsslein-Volhard, 1986) describe the embryonic stages). In the relatively simple stage 9 ectodermal epithelium, Armadillo stripes occupy half to two-thirds of the cells of each segment. Stripes appear graded at their edges, precluding precise delineation of their boundaries, but double labeling with antibodies to Armadillo and Engrailed allowed positioning of stripes within the segment. Armadillo stripes overlap in part the Engrailed-expressing cells, with the center of each stripe just anterior to cells expressing Engrailed, and the posterior margin including most but not all Engrailed-expressing cells (Fig. 2A-C). Armadillo stripes thus center on wg-expressing cells (van den Heuvel et al., 1989; Gonzalez et al., 1991), consistent with the role of wg in Armadillo stripe generation (Riggleman et al., 1990; results below).

In most cell types, Armadillo is enriched near the cell membrane, accumulating all along lateral cell-cell boundaries, with an enrichment in the adherens junction at the apical/lateral interface. Cells also have cytoplasmic Armadillo. In stage 9 embryos, two differences between stripe and interstripe cells are apparent (Figs 1B, 2B). First, there is a dramatic difference in the apparent fraction of cytoplasmic Armadillo in stripe versus interstripe cells. In interstripe cells, nearly all Armadillo is found near the cell membrane, similar to Armadillo distribution in many differentiated tissues (Riggleman et al., 1990; Peifer and Wieschaus, 1990). In contrast, in stripe cells much of the Armadillo is cytoplasmic, though Armadillo is still largely enriched at the cell membrane. We confirmed this using alternate fixation conditions that use heat and methanol as fixatives; these conditions appear to wash out loosely bound Armadillo, allowing only the most tightly bound protein to be visualized. While Armadillo in adherens junctions remains, most cytoplasmic Armadillo is lost (Fig. 1D). This dramatically diminishes Armadillo stripes, suggesting that they result largely from differences in accumulation of loosely bound Armadillo.
In addition to apparent differences in intracellular localization, there also are differences in overall intensity of Armadillo staining between stripe and interstripe cells. Stripe cells are about twice as bright as interstripe cells (Fig. 2D). The largest difference is in cytoplasmic staining, though there are also differences in apparent levels of membrane-associated Armadillo. This may involve membrane-associated Armadillo not assembled into adherens junctions; staining in these junctions (Fig. 1G) appears relatively uniform across the segment. During later stages, Armadillo stripes become more complex, paralleling evolution of wg stripes (Riggleman et al., 1990), and the embryo’s morphology also becomes more complex, complicating analysis of Armadillo accumulation. As a result, we focused on stage 9 embryos, when the ectoderm is still a rather simple epithelium.

One trivial model for stripe generation is that periodic alteration in cell shape might give the appearance of segmental periodicity of Armadillo. To rule this out, we collected serial optical sections through the length of the ectodermal cells. While periodic cell shape changes are seen, Armadillo stripes are visible in each section, rendering it unlikely that cell shape changes are their cause (Fig. 1H-K). Moreover, Armadillo stripes extend throughout the ectoderm, neuroblast and mesodermal layers of the germ band (Fig. 1F), consistent with the role of wg in development of all three tissues (Immerglück et al., 1990; Chu-LaGraff and Doe, 1993).

The presence or absence of Armadillo stripes predicts cell fate decisions

In wild-type embryos, Armadillo ‘stripe cells’ receive wg signal, adopt posterior fates and secrete naked cuticle. In contrast, Armadillo ‘interstripe cells’ receive low or zero levels of wg signal, adopt anterior fates and secrete denticles. Generation of Armadillo stripes is dependent on wg (Riggleman et al., 1990). Confocal microscopy revealed that all cells in wg mutants have a pattern of Armadillo accumulation resembling that of wild-type interstripe cells (Fig. 2E), with primarily cell surface Armadillo and slightly reduced levels of overall Armadillo staining. All surviving cells in a wg mutant will adopt anterior fates and secrete denticles.

The correlation between Armadillo accumulation and cell fate was further tested using embryos mutant for the maternal effect segment polarity gene zeste-white 3 (zw3). The zw3 mutant phenotype is complementary to that of wg; all surviving cells adopt posterior fates and secrete naked cuticle (Perrimon and Smouse, 1989). zw3 is the homolog of vertebrate glycogen synthase kinase-3β (GSK-3β; Siegfried et al., 1992; de Groot et al., 1993) and is required for wg signal transduction (Siegfried et al., 1992). We examined Armadillo striping in embryos lacking functional zw3. No striped patterns were observed. Instead, Armadillo accumulates to higher levels in...
all cells and cytoplasmic staining is almost as intense as membrane staining (Fig. 3A-C). These differences were apparent at the blastoderm stage, as soon as cells form (Fig. 3H-J). The uniform increased staining of zw3 mutant embryos mimics the accumulation observed in the stripes of wild-type embryos. Once again, Armadillo accumulation parallels cell fate. A similar alteration in levels of Armadillo was observed in embryos manipulated so that all cells see high Wingless levels (Noordemeer et al., 1992).

Consistent with the maternal dependence of its phenotype, zw3 affects Armadillo stripes early and alterations are not observed in embryos derived from heterozygous mothers. Although all embryos lacking maternal zw3 die with a naked cuticle phenotype, phenotypic severity is slightly alleviated by zygotic zw3+ (Perrimon and Smouse, 1989). We used linked genetic markers to distinguish embryos zygotically mutant for zw3 from those receiving zygotic zw3+, and found that zygotic rescue is visible in the Armadillo accumulation pattern. Although all embryos show high levels of cytoplasmic staining at the onset of gastrulation, Armadillo staining in zw3/+ heterozygotes is detectably altered by mid-stage 9 (Fig. 3K-L). High levels of cytoplasmic staining decrease in zygotically wild-type embryos, but clumps of intensely staining cells remain.

**Armadillo stripes result from alteration in intracellular protein distribution**

Three possible mechanisms may contribute to generation of Armadillo stripes. The first and least likely was that wg signal triggers a conformational change in Armadillo, altering recognition by our antibody. To test this, we used several monoclonal antibodies to different regions of both the N and C terminus; all recognize Armadillo stripes (Fig. 1C, E; data not shown).

Fig. 2. Armadillo stripes, Engrailed and wingless. All embryos are stage 9. (A-C) Armadillo and Engrailed stripes overlap. Wild-type embryos stained with antibodies to Armadillo (rhodamine-linked secondary antibody) and to Engrailed (FITC-linked secondary antibody). Anterior is to the right. (A) Both antibodies; (B) Armadillo alone; (C) Engrailed alone. Armadillo stripes include anterior Engrailed-expressing cells (e.g., arrowhead) but not all posterior ones (e.g., arrow). (D) False color image of wild-type embryo stained with anti-Armadillo antibody. The brightness of each pixel was assigned a color value: 0-10=black, 10-60=blue, 61-140=green, and 141-250=red. Both membrane and cytoplasmic staining are brighter in stripe cells, but differences in cytoplasmic staining are of a greater magnitude. (E) Equivalent false-color image of a wg mutant. All cells look like wild-type interstripe cells.
shown). Barring total alteration of Armadillo structure, the altered conformation model is thus unlikely.

Visual inspection of antibody-stained embryos suggests that the striped appearance of Armadillo may reflect a combination of changes in total Armadillo levels, such that stripe cells contain more Armadillo than interstripe cells, and alteration of Armadillo intracellular distribution in stripe versus interstripe cells. Staining patterns can, however, be affected by accessibility to antibody, and are thus not strictly quantitative. We took a more direct approach to evaluate relative contributions of these two processes to Armadillo stripe generation. Although we cannot directly compare levels and intracellular distribution of Armadillo in stripe and interstripe cells, we took advantage of the altered staining patterns of wg and zw3 mutants to compare wild-type embryos (with both stripe and interstripe cells), zw3 embryos (entirely composed of apparent stripe cells) and wg embryos (entirely apparent interstripe cells).

In a wild-type embryo, about three-quarters of the Armadillo is membrane-associated and the other quarter is cytoplasmic (Peifer, 1993). We compared ratios of cytoplasmic versus membrane-bound Armadillo in wild-type, wg and zw3 embryos. Wild-type or mutant embryos were fractionated into membrane-associated (P100) and cytoplasmic (S100) fractions, and the fractions evaluated by SDS-PAGE and immunoblotting (Fig. 4), using the soluble protein Bicaudal D as a control. Wild-type, wg and zw3 embryos differ substantially in levels of cytoplasmic Armadillo. wg mutant embryos have less cytoplasmic Armadillo than wild type, while zw3 mutants have substantially more cytoplasmic Armadillo (Fig. 4), consistent with our immunofluorescence data (Figs 1-3).

In parallel we compared the total amount of Armadillo in wild-type, wg or zw3 mutants to evaluate whether stripes reflect segmentally repeated differences in Armadillo levels. Subtle but reproducible differences in levels of Armadillo in wild-type, wg and zw3 embryos were seen (Fig. 5). At stage 9
when stripes are most prominent, \(wg\) mutants contain about three-quarters the wild-type level of Armadillo, while \(zw3\) mutant embryos contain one-and-a-half to two times as much. These results together with those above suggest that while wild-type, \(wg\) and \(zw3\) mutant embryos have similar levels of membrane-associated Armadillo, \(wg\) mutations decrease the amount of cytoplasmic Armadillo while \(zw3\) mutations increase cytoplasmic Armadillo. The difference in Armadillo levels between \(zw3\) and wild-type embryos is most extreme early and becomes less pronounced with time (Fig. 5), as expected since half of the \(zw3\) mutant embryos receive \(zw3^+\) from their father. Zygotic \(zw3\) mutant embryos are derived from mutant germ cells; half of these embryos receive a wild-type gene from their father. (Left set of panels) Amounts of Armadillo in wild-type and \(zw3\) mutants. Levels of Armadillo are elevated in \(zw3\) mutant embryos before the blastoderm stage and remain elevated throughout. (Right set of panels) Amounts of Armadillo in wild-type and \(wg\) mutants. Levels of Armadillo are slightly reduced relative to wild-type. Numbers indicate number of embryos homogenized.

### armadillo function appears to be required for Armadillo stripe generation

\(arm\) mutations that block \(wg\) signaling might be expected to block Armadillo striping. We examined this using mutations that truncate Armadillo but make detectable, though reduced, levels of truncated protein (Peifer and Wieschaus, 1990). Antibodies against Armadillo’s N-terminal domain recognize wild-type and mutant Armadillo, while anti-C-terminal antibodies recognize only wild-type protein. Due to the \(arm\) maternal contribution, one must make homozygous mutant germ-line clones to produce embryos completely lacking wild-type Armadillo. This is only possible with moderate \(arm\) alleles; severe alleles block oogenesis (Peifer et al., 1993). Embryos maternally and zygotically mutant for a moderate \(arm\) allele are identical in phenotype to null \(wg\) mutants (Klingensmith et al., 1989).

We generated embryos with no residual wild-type maternal Armadillo, yielding two classes of progeny, half receiving a wild-type \(arm\) gene from their father (XX females) and half that do not (XY males). To distinguish these classes of embryos, we linked the mutation \(twisted\) gastrulation to \(arm\), allowing zygotic detection of \(arm\) mutant embryos by their
failure to gastrulate correctly. Since the maternal Armadillo is the truncated mutant protein, all embryos initially fail to stain with C-terminal antibodies. Half the embryos (those receiving a wild-type paternal arm) produce wild-type zygotic Armadillo; soon after the onset of gastrulation wild-type protein is observed in these embryos (detected by C-terminal antibodies).

Loss of arm function correlates with the failure to form Armadillo stripes. Embryos with a wild-type zygotic gene accumulate Armadillo in stripes, similar to though less intense than a wild-type embryo (Fig. 6A,C). Because we lack antibodies specifically recognizing mutant proteins, we cannot determine whether mutant protein accumulates (along with wild-type protein) in these stripes. In contrast, stripes are never seen in embryos lacking both maternal and zygotic arm (Fig. 6B,D,E); mutant protein assumes a distribution like that in wild-type interstripe cells (Fig. 6G-I), as Armadillo does in wg mutants. These results are consistent with a role for arm function in Armadillo stripe generation, but it is also possible that the protein products produced by these two mutant alleles are resistant to whatever mechanism generates stripes.

We examined whether Armadillo proteins lacking the C-terminal domain localize appropriately within the cell. armH8.5 protein localizes to the cell surface in the embryonic epidermis (Fig. 6H), and, like wild-type Armadillo (Peifer, 1993; Fig. 1G), armH8.5 protein is enriched in the region of the adherens junctions (data not shown), consistent with its behavior in larval tissues (Peifer and Wieschaus, 1990). arm25B protein is similarly localized (Fig. 6G), but the precise nature of the protein disruption in this allele is unclear (Peifer and Wieschaus, 1990). The level of mutant protein accumulating in the more severe allele armXM19 is substantially lower than normal (Fig. 6E), but this protein is enriched in the region of the membrane (Fig. 6I). Thus, the Armadillo C-terminal domain is not critical for membrane-association or assembly into adherens junctions.

**arm functions downstream of zw3 in the wg signaling pathway**

The results above suggest that arm might act downstream of both wg and zw3 in wg signaling. To evaluate this, we examined arm zw3 double mutants. As arm and zw3 have opposite effects on cell fate, double mutant analysis allows one to order these mutations in the wg signaling pathway. Since both zw3 and arm are provided both maternally and zygotically, we used FLP-site-specific recombination to make female germ-line clones homozygous for mutations in both arm and zw3. Both genes are on the X chromosome, so when females with mutant germ lines are crossed to wild-type males, two classes of progeny result. Half receive a paternal Y chromosome and thus have wild-type zygotic Armadillo; soon after the onset of gastrulation wild-type germ-line clones fall into two distinct classes present at a 1:1 ratio, which thus represent embryos that either did or did not receive wild-type paternal genes. The first class, embryos lacking maternal and zygotic zw3 and arm function (Fig. 7E,F), have an embryonic cuticle phenotype like that of arm alone (Fig. 7D), strongly suggesting that arm acts downstream of zw3. A similar epistasis was observed in the effect on Engrailed staining. arm zw3 double mutants (data not shown) do not show the expanded Engrailed expression domain of zw3.
embryos (Siegfried et al., 1992), but instead show loss of Engrailed as seen in arm mutants (Peifer et al., 1991). arm zw3 double mutants were also stained with anti-Armadillo antibodies. Armadillo staining patterns of armXM19 zw3 and armH8.6 zw3 double mutants were indistinguishable from those of armXM19 and armH8.6 single mutants (Fig. 6I; data not shown).

These phenotypes are only seen in half of the progeny. The other embryos receive wild-type paternal zw3 and arm. Since zygotic arm rescues embryos lacking maternal arm (Wieschaus and Noell, 1986), these embryos should not show the arm phenotype, but since zygotic zw3 does not rescue embryos lacking maternal zw3 (Perrimon and Smouse, 1989), zygotically ‘rescued’ double mutants might be expected to secrete mostly naked cuticle. Surprisingly, however, arm zw3/+ + progeny derived from arm zw3 germ-line clones do not show the zw3 naked cuticle phenotype. Instead, they develop into normal embryos (Fig. 7G) that survive to adulthood. Similar results were obtained with three different arm alleles. The only difference between surviving animals maternally mutant for arm and zw3 and ‘naked cuticle’ embryos maternally mutant for zw3 alone is absence of maternal arm. Removal of maternal arm completely suppresses the maternal effect of zw3. This suggests that all effects of the loss of maternal zw3 are mediated through effects on arm function. In arm zw3/+ + progeny derived from arm zw3 germ-line clones, arm+ is supplied zygotically, but simultaneously, with zw3+. To demonstrate that zygotic zw3 is required for rescue, we crossed arm zw3 germ-line mosaics to arm mutant males carrying a Y chromosome duplication of arm+ but not zw3+. generating embryos lacking maternal arm and zw3 that receive zygotic arm+ but not zygotic zw3+.

Fig. 7. arm is downstream of zw3 in the wg signaling pathway. Cuticle preparations of embryos derived from zw3, arm, and arm zw3 germ-line clones. (A) Wild-type embryo. Note alternating denticles (arrows) and naked cuticle (arrowheads). (B) Putative zw3/Y embryo derived from zw3 germ-line clone, with entirely naked cuticle. (C) Putative zw3/+ embryo from zw3 germ-line clone. Note occasional patches of denticles (e.g. arrow). (D) armXM19/Y embryo from armXM19 germ-line clone; all cells secrete denticles. (E) armH8.6 zw3/Y embryo from armH8.6 zw3 germ-line clone; embryos show the arm mutant phenotype, though these embryos have a slightly weaker segment polarity phenotype than those from armH8.6 single mutant germ-line clones. This slight rescue may reflect the fact that armH8.6 protein retains a small amount of function. Maternally contributed mutant Armadillo, in the absence of maternal zw3, appears to act to promote naked cuticle to a slight extent, alleviating the arm mutant phenotype slightly. (F) armXM19 zw3/Y embryo from armXM19 zw3 germ-line clone; all cells secrete denticles. (G) armXM19 zw3/+ embryo from armXM19 zw3 germ-line clone; this type of embryo develops to adulthood. (H) armXM19 zw3/arm1035 zw3+ embryo derived from armXM19 zw3 germ-line clone; zygotic arm is necessary for rescue. (I) armXM19 zw3/Y arm+ embryo derived from armXM19 zw3 germ-line clone; zygotic zw3 is necessary for rescue.
Arm stripes and \( wg \) signal transduction

not rescued but instead show the \( zw3 \) naked phenotype (Fig. 7I). Likewise, zygotic \( arm \) is also required for rescue (Fig. 7H). Thus maternal effect of \( zw3 \) can be suppressed by removing maternal \( arm \), as long as both \( zw3 \) and \( arm \) are provided zygotically. Similar results were obtained with three different \( arm \) alleles, and this epistasis has also been independently observed by Siegfried et al. (1993).

**DISCUSSION**

Pattern formation within the *Drosophila* embryonic segment provides an example of how cell-cell signaling affects cell fate. Wingless is a cell-cell signaling molecule required for pattern formation within each segment (reviewed in Peifer and Bejsovec, 1992). Other segment polarity genes may encode components of the machinery to secrete, receive and transduce \( wg \) signal. We focus on \( armadillo \) (\( arm = \) gene; Armadillo = protein), which is required for cells to respond to \( wg \) (Wieschaus and Riggleman, 1987; Peifer et al., 1991). This is surprising, since Armadillo is the structural and functional homolog of the vertebrate cell-cell adhesive junction protein \( \beta \)-catenin (McCrea et al., 1991; Peifer et al., 1993; Peifer, 1993; Oda et al., 1993). This raised two possibilities. Armadillo and adherens junctions might be indirectly responsible for \( wg \) signaling via effects on localization of the putative Wingless receptor. Alternately, Armadillo might play an unexpected direct role in \( wg \) signaling. We attempted to resolve these two possibilities.

**The intracellular distribution of Armadillo predicts cell fate**

Although \( arm \) RNA is expressed at high levels in all embryonic epidermal cells (Riggleman et al., 1989), Armadillo accumulates in segmentally repeated stripes (Riggleman et al., 1990; Figs 1,2). This is one of the first cellular responses to Wingless (Riggleman et al., 1990; Fig. 2). We examined this connection in detail. In most cells, \( \geq 75\% \) of the Armadillo is membrane-associated as part of the adherens junction (Peifer, 1993). This intracellular distribution of Armadillo is altered in response to Wingless. Within each segment, wild-type embryos have posterior Armadillo stripes and anterior interstripes. In response to \( wg \), stripe cells accumulate substantially higher levels of cytoplasmic Armadillo. Interstripe cells, receiving little or no \( wg \), retain primarily membrane-associated Armadillo. This difference in Armadillo intracellular localization is later reflected in different cell fate choices; stripe cells

---

**Fig. 8.** Two models for Armadillo function in \( wg \) signal transduction. See text for details.
choose posterior fates and secrete naked cuticle, and interstripe cells choose anterior fates and make denticles.

This correlation is intriguing; Armadillo stripes appear to be a direct visualization of wg signal transduction. This is supported by two other observations. In wg mutants, lacking wg signal and in which all surviving cells adopt an anterior fate, Armadillo stripes never form. Armadillo remains membrane-associated in all cells (Fig. 2E), as it would in interstripe cells in a wild-type embryo. In contrast, in zw3 mutants that behave as if wg signaling was uniformly hyperactivated, all surviving cells adopt a posterior fate and all cells have substantial cytoplasmic Armadillo (Fig. 3), as normal stripe cells would. Armadillo stripes reflect and may be a necessary step in transduction of wg signal.

In addition to changing Armadillo intracellular distribution (Fig. 4), wg and zw3 mutations also alter total cellular levels of Armadillo (Fig. 5). One alteration may be primary and the other a secondary consequence. If alterations in intracellular distribution are primary, changes in Armadillo levels could result from differences in Armadillo stability in different cellular compartments. In contrast, if alterations in amount of Armadillo are primary, one might imagine that there are a saturable number of sites of membrane-association, and thus increasing levels of Armadillo might specifically increase the cytoplasmic form. The similar levels of membrane-associated Armadillo in wild-type, wg mutant and zw3 mutant embryos (Fig. 4) make it most likely that the observed changes in overall levels result largely from dramatic changes in levels of cytoplasmic Armadillo. In fact, absolute Armadillo levels do not seem critical in generating segmental pattern. Altering copy number of the arm gene from one to four has no effect (unpublished data). Likewise, armH8.6 mutants pass through embryogenesis normally at 18°C even though their levels of mutant protein are substantially lower than those of wild-type protein in a wild-type embryo (Peifer and Wieschaus, 1990).

**Armadillo plays a direct and essential role in transmission of wg signal**

The role of arm in wg signaling could be direct or indirect. Our data suggest that wg signal triggers an alteration in Armadillo intracellular distribution, consistent with a direct role. To demonstrate a causal connection, we used genetic epistasis analysis to position arm within the wg signaling pathway. If two mutations have opposite phenotypes, the double mutant phenotype reveals the position of the two mutations relative to each other in a genetic pathway. For example, Siegfried et al. (1992) showed that zw3; wg double mutants have the ‘naked cuticle’ phenotype of zw3 mutants rather than the ‘all denticle’ phenotype of wg. They proposed that zw3 is downstream of wg and that wg negatively regulates Zeste-white-3 kinase activity.

Epistasis tests allowed us to position arm with respect to zw3 in this pathway (Fig. 7). If arm and adherens junctions were involved indirectly (e.g. for correct function or localization of the putative Wingless receptor) arm would be indistinguishable from wg in epistasis with zw3. This is not what we found. Instead, arm zw3 double mutants have the ‘all denticle’ phenotype of arm rather than the ‘naked cuticle’ phenotype of zw3. Similar epistasis results have been independently obtained by Siegfried et al. (1993). Formally, this positions arm downstream of zw3 and suggests that zw3’s role in signaling is mediated at least in part by effects on arm. Consistent with this, zw3 encodes a kinase, Armadillo is phosphorylated and its phosphorylation is dramatically reduced in zw3 mutants (Peifer et al., unpublished data). Armadillo may be an indirect or perhaps even a direct target of Zeste-white 3 kinase.

**Molecular models for the role of Armadillo in wg signaling**

The data presented above support the idea that generation of Armadillo stripes is not just a consequence of wg but a necessary step in signal transduction. This leaves the question of mechanism. wg signal stimulates an increase in levels of cytoplasmic Armadillo. Given this, it seems plausible that cytoplasmic Armadillo interacts with an effector to mediate wg signal transduction. Zeste-white 3 normally acts counter to this, keeping levels of cytoplasmic Armadillo relatively low and preventing signal transduction in the absence of wg signal. Any model for the role of cytoplasmic Armadillo in signaling must address several unanswered questions. First, what is the mechanism by which cytoplasmic Armadillo levels are increased? Second, what are the roles of wg signal and Zeste-white 3 kinase in this process? Third, what is the nature of the effector with which cytoplasmic Armadillo interacts? As a way of envisioning possible mechanisms of Armadillo action, we outlined two of the many models consistent with our results in Fig. 8. In both models, Wingless is instructive, while Zeste-white 3 is a key supporting player. In the following discussion, we will refer to the action of Zeste-white 3 on Armadillo as if it is direct; of course it may be indirectly mediated by one or more intervening factors. The models differ in the way in which they answer the three questions outlined above. Many of the features of the two models could be interchanged with the same ultimate results; they are thus to be viewed as examples to stimulate thought about the process.

In Model 1, levels of cytoplasmic Armadillo increase as a result of an alteration in protein stability. Zeste-white 3 kinase might regulate stability of cytoplasmic Armadillo; phosphorylated protein could have a short half-life. In the absence of zw3, cytoplasmic Armadillo would accumulate. This effect would be mimicked by wg signal, perhaps by inhibition of Zeste-white 3 activity or by activation of a phosphatase. This model proposes that the effector of wg signal is the adherens junction complex itself, emphasizing Armadillo’s known role in this structure. Cells receiving wg signal accumulate increased cytoplasmic Armadillo, driving more Armadillo into junctional complexes at the cell surface, consistent with subtle increases seen in cell surface-staining in stripe cells versus interstripe cells. Though quantitatively small, such changes might have major consequences on juxtaposition of constitutive ligands and receptors or on cytoskeletal organization. Spatial patterning of the epidermis would be preceded and mediated by local changes in adhesion regulated by wg signal. Such hypothetical changes in adhesion would mediate all consequences of wg, including effects on gene expression.

In Model 2, the source of cytoplasmic Armadillo is the pool of Armadillo normally assembled into junctions. In the absence of wg, virtually all cellular Armadillo is part of the membrane-associated adherens junction, comprised of Armadillo, a glycoprotein that is likely a cadherin homolog (by analogy to the vertebrate adherens junction), and the *Drosophila* homolog of
α-catenin (Peifer, 1993; Oda et al., 1993). In this model, Zeste-white 3 kinase would maintain efficient assembly of junctional complexes, keeping normal levels of cytoplasmic Armadillo relatively low. Wingless signal is envisioned to stimulate disassembly of a subset of Armadillo-containing junctional complexes, releasing Armadillo into the cytoplasm. In an extreme version of this model, the putative Armadillo-associated cadherin might even function as the Wingless receptor. At least half of the cellular Armadillo remains in the adherens junction — the pool of membrane-associated Armadillo is large, so relatively subtle changes in occupancy of adherens junctions could raise relative levels of cytoplasmic Armadillo substantially. wg-stimulated release of Armadillo from junctional complexes may or may not involve alterations in Armadillo phosphorylation. Cytoplasmic Armadillo then activates a cytoplasmic effector (such as a kinase), further transducing wg signal. Either cytoplasmic Armadillo or junctional complex lacking Armadillo could potentially stimulate signal transduction. These are not mutually exclusive alternatives. G-proteins that couple membrane receptors to intracellular effectors are composed of three subunits; both α and β subunits of the G-protein complex interact with separate effectors (reviewed in Birnbaumer, 1992). Both cytoplasmic Armadillo and junctional complex lacking Armadillo could potentially interact with separate effectors. Since reduction in Armadillo levels in arm mutants disrupts wg signaling, however, cytoplasmic Armadillo, rather than disassembled junctional complex, appears to be rate-limiting. As mentioned above, individual components of these two models are interchangeable. For example, aspects of both models could be combined to suggest that wg signal and Zeste-white 3 kinase regulate stability of cytoplasmic Armadillo, increasing its levels and stimulating a cytoplasmic effector.

If zw3 acts via arm, zw3 elimination ought have no consequences if arm is also absent; this is observed in the arm zw3 double mutant. Our results support an even stronger statement. Given the apparent role of zw3 in other signaling pathways, such as that downstream of Notch (Heitzler and Simpson, 1991), and the broad specificity in vitro of its homolog GSK-3β, it is likely that zw3 has other cellular targets. However, our analysis suggests that in the context of wg signaling, Armadillo may be its key and perhaps only target. Embryos lacking maternal zw3 are not rescued by wild-type zygotic zw3. There are two possible explanations for this. One is that without maternal zw3, a sufficient level of embryonic zw3 function cannot be achieved. This is not true, however; embryos derived from mothers lacking both zw3 and arm are rescued by zygotic gene expression. Loss of maternal zw3 has consequences during oogenesis that can be relieved by simultaneously removing arm. In the absence of maternal zw3, dephosphorylated Armadillo (Peifer et al., unpublished data) is deposited into eggs and this cytoplasmic Armadillo is present when wg signaling begins (Figs 3, 5). This cytoplasmic Armadillo would, in our models, stimulate all cells to act as if they had received wg signal, leading to irreversible incorrect cell fate choices. If the mother lacks both zw3 and arm, she deposits no Armadillo into her eggs. When zygotic gene expression begins, both zw3 and arm are made, leading to synthesis of correctly phosphorylated and localized Armadillo, and a normal phenotype.

**A specific role for the C-terminal domain of Armadillo in wg signaling**

Armadillo protein can be divided into three regions, thirteen imperfect 42 amino acid repeats making up the central two-thirds, an acidic N-terminal region and a glycine-rich C terminus (Riggleman et al., 1989). These ‘domains’ are conserved to varying degrees in Armadillo’s homologs. We believe Armadillo acts as an adapter, analogous to SH2/SH3 proteins, connecting one molecule to another. Different domains may mediate specific protein-protein interactions. Structural and functional studies (Peifer, 1993; Peifer et al., 1993) suggest that Armadillo is the β-catenin homolog, required for cell adhesion and cytoskeletal function; Armadillo is also required for wg signal transduction. It is possible that these functions are separable, such that given domains of Armadillo might be required for one function and not the other.

The experiments above, along with previous work, raise the possibility that Armadillo’s C-terminal domain is critical for wg signaling and less important for junctional function. While all arm alleles disrupt wg signaling (Peifer and Wieschaus, 1990; Peifer et al., 1991; results above), alleles differ in their effects on adherens junction function. Proteins encoded by armHβ6 or armXM19, lacking the C-terminal domain but retaining the repeats, are sufficient for cell adhesion and cytoskeletal integrity during oogenesis (Peifer et al., 1993), while alleles removing in addition significant portions of the repeat region disrupt oogenesis (Peifer et al., 1993). Armadillo’s N-terminal and repeat regions appear necessary and sufficient for Armadillo’s adhesive function but are not sufficient for wg signaling. The C-terminal domain may play a special role in wg signaling, for example regulating release from the membrane in response to wg, or interacting with a cytoplasmic effector.

**wnt proteins regulate developmental decisions in vertebrates** (McMahon, 1992). Is the entire wg signaling system conserved? A decisive answer will require mutation of β-catenin and GSK-3β, but there is an intriguing indication that similar machinery is used to transmit wt signal. Injection of anti-β-catenin antibody into Xenopus embryos leads to dorsal axis duplication (McCrea et al., 1993) very similar to that produced by wtRNA injection (e.g. McMahon and Moon, 1989). Further, Bradley et al. (1993) have demonstrated an effect of Wnt-1 on accumulation of the other Armadillo homolog plakoglobin and on accumulation of E-cadherin in vertebrate tissue culture cells and have further shown that this change alters cell adhesive properties. These results together suggest that Armadillo/β-catenin/plakoglobin are involved in response to wgt wt signal in insects and in vertebrates. The next challenge is to identify the biochemical role of Armadillo/β-catenin in signal transduction.

We are grateful to E. Siegfried, T.-B. Chou and N. Perrimon for providing fly stocks, sharing and discussing results before publication, B. Gumbiner and P. McCrea for sharing results before publication, B. Suter for anti-Bicaudal D antibody, D. Mielenicki for able technical assistance, S. Whitfield for skilled photography, A. Bejsovec, H. Harkins, C. A. McCormick and the two reviewers for helping clarify both our thinking and the manuscript, and members of the Peifer, Wieschaus and Searle labs for discussions. This work was supported by NIH grants to M. P. (GM47857) and to E. W. (HD22780), and by a grant from the Searle Scholars Program to M. P.
REFERENCES


(Accepted 14 October 1993)