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A model system for cell adhesion and signal transduction in *Drosophila*

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SUMMARY

Cells must cooperate and communicate to form a multicellular animal. Information about the molecules required for these processes have come from a variety of sources; the convergence between the studies of particular molecules by vertebrate cell biologists and the genes identified by scientists investigating development in *Drosophila* has been especially fruitful. We are interested in the connection between cadherin proteins that regulate cell-cell adhesion and the *wingless/wnt-1* cell-cell signaling molecules controlling pattern formation during development. The *Drosophila* segment polarity gene *armadillo*, homolog of the vertebrate adherens junction protein β-catenin, is required for both cell adhesion and *wg* signaling. We review what is known about *wingless* signaling in *Drosophila*, and discuss the role of cell-cell junctions in both cell adhesion and cell communication. We then describe the results of our preliminary structure-function analysis of Armadillo protein in both cell adhesion and *wingless* signaling. Finally, we discuss evidence supporting a direct role for Armadillo and adherens junction in transduction of *wingless* signal.

Key words: *wingless*, *armadillo*, pattern formation, cadherin, cell-cell adhesion

INTRODUCTION

Fifteen years have passed since Eric Wieschaus and Christiane Nüsslein-Volhard initiated their screen for zygotic lethal mutations affecting the cuticular pattern of the *Drosophila* embryo (Nüsslein-Volhard and Wieschaus, 1980; Wieschaus et al., 1984; Nüsslein-Volhard et al., 1984; Jürgens et al., 1984). In the last 10 years many of the ~120 genes they identified have been examined in detail, and the results are both revealing and surprising. These genes encode a wide variety of proteins involved in an impressive array of cellular processes. Initial attention was focused on those encoding transcription factors; many form the zygotic cascade of gene products that set up the segmental pattern. However, recently attention has turned to genes involved in other cellular processes: cell cycle control (e.g., *string* encodes the *Drosophila* homolog of fission yeast *cdc25*; Edgar and O'Farrell, 1989), cell-cell signaling (e.g., *faint little ball* encodes the *Drosophila* EGF receptor; Price et al., 1989; Schejter and Shilo, 1989), cytoskeletal function (e.g., *zipper* encodes cytoplasmic myosin heavy chain; Young et al., 1993), and establishment of apical-basal polarity (*crumbs* is required to generate epithelial cell polarity; Tepass et al., 1990). These genes provide an indication of the potential of the *Drosophila* system for studying virtually any cell biological question – one can combine molecular tools, in vivo cell biology, and genetic analysis.

The connections between genes involved in *Drosophila* pattern formation and proteins identified by vertebrate cell biologists have been extremely fruitful for the advance of knowledge in both fields. We will focus on the recently identified connection between the cadherin family of cell adhesion molecules that form cell-cell adhesive junctions and the *wingless/wnt* family of cell-cell signaling molecules that control pattern formation in insects and vertebrates. A variety of experiments underway simultaneously with those of Wieschaus, Nüsslein-Volhard, and colleagues contributed to this connection. In Japan, Masatoshi Takeichi and his colleagues characterized cadherins as the molecules responsible for Ca^{2+}-dependent adhesion in mammalian cells (Takeichi, 1977; Takeichi et al., 1979; Urishihara et al., 1979); the same molecules were independently shown to be required for mouse embryogenesis by Rolf Kemler and others in the laboratory of François Jacob in Paris (Kemler et al., 1977; Peyriéras et al., 1985). In India and England, labs were investigating the effects of the *Drosophila wingless* mutation on cell communication and cooperation during development of the adult body (Sharma and Chopra, 1976; Morata and Lawrence, 1977). *wingless* would prove to be the *Drosophila* homolog of *wnt-1*, identified by Roel Nusse as one of the genes activated by MMTV in murine mammary tumors (Nusse and Varmus, 1982). The connection between the regulation of cell-cell adhesion and the function of the *wingless/wnt-1* cell-cell signaling system was made by the recognition that one molecule was required for both processes, the *Drosophila* Armadillo protein, homolog of vertebrate β-catenin. This connection provided one of the links that established the key role of cell-cell and cell-matrix junctions in mediating communication and cooperation among cells.

CELL SIGNALING AS A REGULATOR OF CELL FATE

Regulation of the number and identity of each embryonic segment in *Drosophila* is under the control of transcription factors that act in a hierarchical fashion to provide positional information to each of the embryo's cells. However, while these cell-intrinsic cues control segment number and identity, further elaboration of the pattern within each segment requires cell-cell interactions regulated by products of the segment polarity genes (Nüsslein-Volhard and Wieschaus, 1980). This was first revealed by the properties of the *wingless (wg)* gene; it operates in a cell-cell communication process required to set up pattern within each segment (Morata and Lawrence, 1977). The *armadillo* gene (*arm* = gene; Armadillo = protein; Wieschaus et al., 1984) is another one of the group of segment polarity genes.

The intrasegmental pattern is easiest to visualize on the ventral surface of the embryo (Fig. 1). Within each segment, anterior cells secrete small hairs called denticles, while posterior cells secrete naked cuticle devoid of denticles. In fact, each of the approximately twelve rows of cells present within each segment at the end of embryogenesis has a unique fate. Within the denticle belt, denticles differ in shape, size and orientation from front to back; within the naked cuticle region, cells in particular anterior-posterior positions produce special sense organs, attach to muscles underneath the epidermal layer, or have other unique properties.

The segment polarity genes are responsible for elaboration of this complex pattern. Many segment polarity mutations yield a similar phenotype, exemplified by that of *wg* or *arm*. In a *wg* or *arm* mutant, all surviving cells make denticles and no cells make naked cuticle (Fig. 1). An explanation for this was provided by molecular analysis of *wg*, which encodes a secreted molecule that is the homolog of the vertebrate oncogene *wnt-1* (Baker, 1987; Rijsewijk et al., 1987; van den Heuvel et al., 1989). *wg/wnt-1* serve as cell-cell signaling molecules. *wg* RNA is expressed in a subset of the cells within each segment (Fig. 2A), but Wingless protein is secreted and, at least early in embryogenesis, assumes a graded distribution across the entire segment. The highest levels of Wingless are present surrounding cells that secrete it, and successively lower levels are found in cells distant from this position (Gonzalez et al., 1991; Fig. 2B,C; reviewed by Peifer and Bejsovec, 1992). Wingless serves as an intercellular signal, as demonstrated by the ability of *wg* to affect the behavior of cells at a distance (Morata and Lawrence, 1977).

It has been suggested that *wg* serves not only as a cell-cell signal, but also as a graded morphogen. It has been proposed that cells are sensitive to the levels of *wg* signal to which they are exposed, and thereby determine their position within the segment and thus their cell fate. This is consistent with certain experimental results. For example, in a *wg* mutant all surviving cells adopt a specific anterior fate and secrete a single type of denticle, as would specific anterior cells of each segment, while in animals in which *wg* has been ectopically expressed at a very high level in all cells, all cells adopt a posterior fate and secrete naked cuticle, as would wild-type cells exposed to the maximum levels of *wg* (Noordermeer et al., 1992). If *wg* is a graded

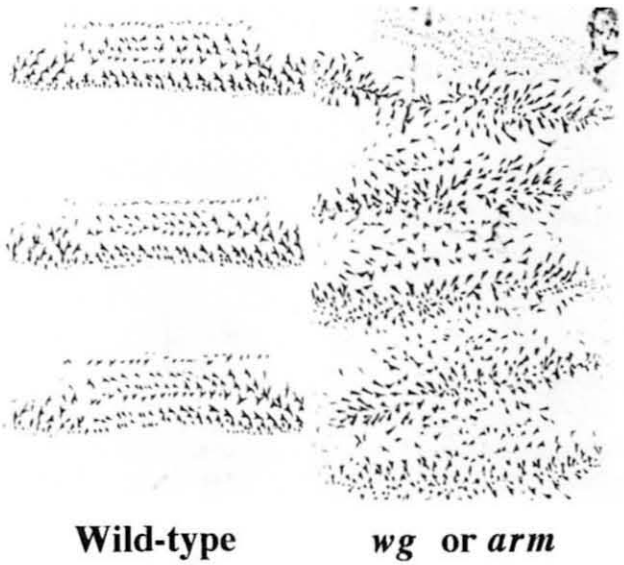


Fig. 1. Segment polarity genes disrupt pattern within each segment. The anterior-posterior pattern of the cuticle is most easily observed on the ventral side of the embryo. Left panel. In a wild-type embryo, anterior cells of each segment secrete small hairs known as denticles, while posterior cells secrete naked cuticle devoid of denticles. The anterior-posterior pattern is even more detailed than this; for example, cells secrete different types and orientations of denticles, depending on their precise position. Right panel. In mutations of the *wingless*-class, such as *wingless* or *armadillo*, all surviving cells secrete denticles, while no cells make naked cuticle.

morphogen, intermediate levels of *wg* ought to lead to cell fates distinct from those conferred by high or low levels; support for a graded role for *wg* in adult development has been obtained by Struhl and Basler (1993).

Some features of this model are controversial, and it is now clear that it is at very least oversimplified. First, *wg* plays different roles at different stages of embryogenesis, acting first to stabilize *engrailed* expression in neighboring cells (Bejsovec and Martinez-Arias, 1991; Heemskerk et al., 1991), and later to promote cell fate diversity along the anterior-posterior axis (Bejsovec and Martinez-Arias, 1991). The graded nature of *wg* signal has been disputed by Lawrence and colleagues (Sampedro et al., 1993), who have shown by a series of clever manipulations that even at uniform levels of *wg* there is still pattern information remaining within the segment. This is consistent with the null phenotype of *wg* mutations; although all cells in a *wg* mutant secrete row 5 denticles, the orientation and spacing of the denticles still varies in a segmentally repeated fashion (Bejsovec and Martinez-Arias, 1991). This remaining pattern information is likely to be conveyed by a subset of the other segment polarity genes, which influence the pattern in part via *wg*-independent mechanisms. Analysis of double and multiple mutant combinations of *wg* and other segment polarity mutations demonstrated that at least *patched*, *hedgehog*, and *naked* operate in this way (Bejsovec and Wieschaus, 1993). The simplest version of the model in Fig. 2B would suggest that different threshold concentrations of *wg* signal would be necessary and sufficient to confer particular cell fates. This is not the case. Cells can adopt a

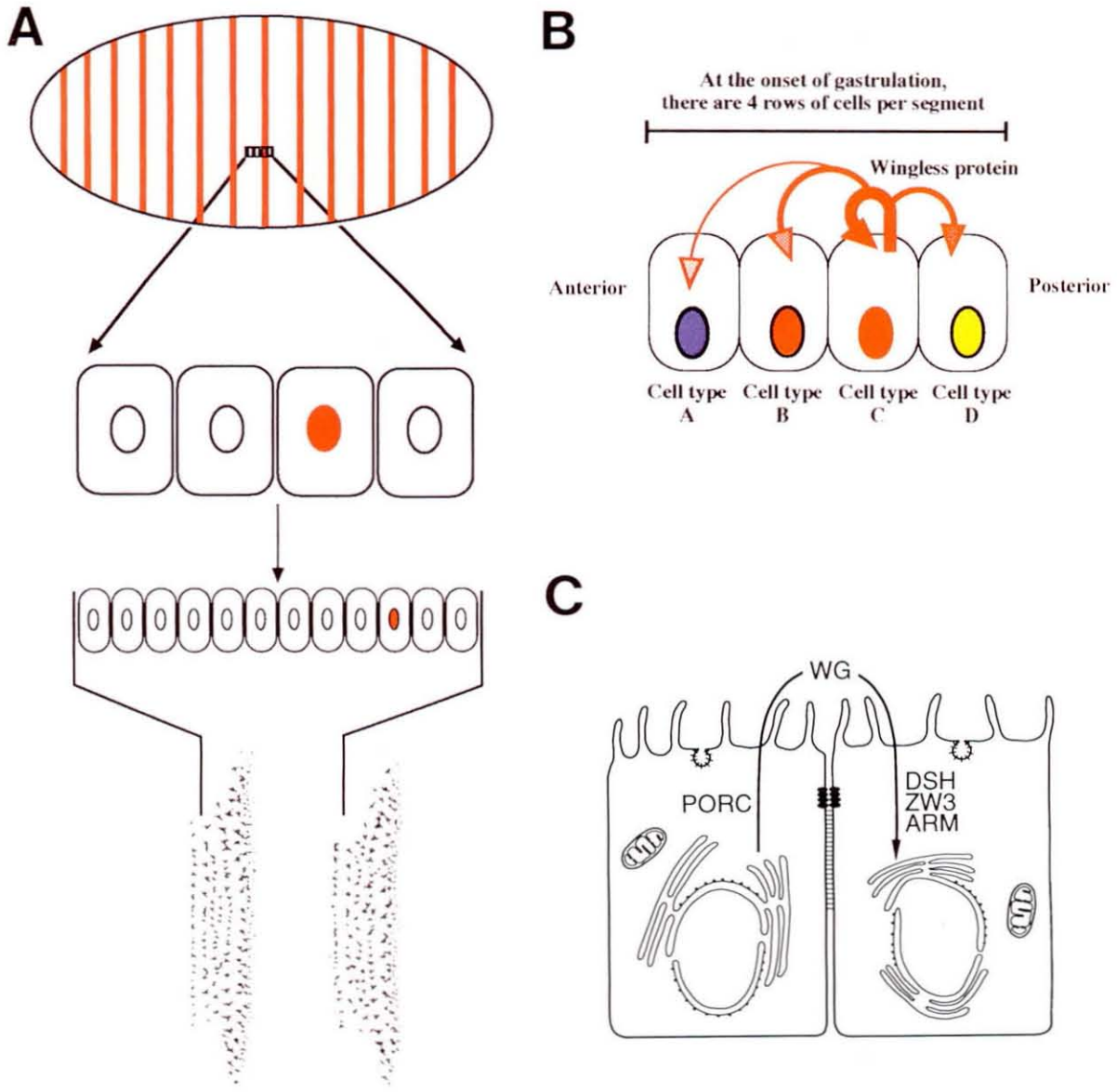


Fig. 2. *wingless* signal regulates cell fate. (A) *wingless* RNA is expressed by a subset of cells in each segment- there is thus one stripe of *wingless* expression per segment (*wingless* RNA expression is indicated in red). At the onset of gastrulation, there are four rows of cells along the anterior-posterior axis in each segment. The third row of cells in each segment express *wingless*. By the end of embryogenesis there will be about twelve rows of cells per segment. The descendants of the *wingless*-expressing cell will produce part of the naked cuticle. (B) The *wingless*-expressing cell secretes Wingless protein, which forms a graded distribution across the segment. Cells may measure this graded *wingless* signal as one of the inputs they use to determine their position within the segment and thus their ultimate cell fate. (C) Other segment polarity genes are thought to encode components of the machinery for producing, receiving, and interpreting *wingless* signal. Genetic analysis suggests that the *porcupine (porc)* gene product is required for production of *wingless (wg)* while *disheveled (dsh)*, *zeste-white 3 (zw3)*, and *armadillo (arm)* are required for signal transduction (see text for details).

variety of different anterior fates (i.e., secrete a variety of denticle types) at a single *wg* concentration, dependent on the action of the other segment polarity genes (Bejsovec and Wieschaus, 1993). The role of *wg* seems to be to create a diversity of cell fates along the anterior-posterior axis (Bejsovec and Wieschaus, 1993). This is still consistent with *wg* serving as a graded signal, but the effect of this signal on ultimate cell fate is modulated, perhaps in a combinatorial fashion, by products of the other segment polarity genes. Much work remains to sort out the interactions required to set up the details of the segmental pattern.

It is clear, however, that *wg* signaling plays a key role in pattern formation, and thus much effort has been expended trying to determine the pathway by which this signal is received and transduced. From the initial screen for zygotic lethals affecting the embryonic pattern and from a subsequent screen for maternal affect mutations affecting this process (Perrimon et al., 1989), a number of genes have been identified with a phenotype similar to that of *wg*. In particular, *arm*, *disheveled*, and *porcupine* are phenotypically identical to *wg*, both in effects on the final cuticular pattern and on more proximate events such as regulation of

engrailed expression (Klingensmith et al., 1989, 1994; Peifer et al., 1991; Theisen et al., 1994). Our working hypothesis is that these genes, and perhaps others sharing phenotypic similarity to *wg*, may encode components of the cellular machinery required to receive and transduce *wg* signal (Fig. 2C). Two different genetic approaches have been used to assess the potential role of various genes in this pathway. First, mutations have been examined to determine whether or not they are cell-autonomous. Cells mutant for genes required for production of functional signal can be rescued by wild-type neighbors, while cells mutant for genes required for reception, transduction, or implementation of signal cannot be rescued. *wg* is non-autonomous – mutant cells are rescued by wild-type neighbors (Morata and Lawrence, 1977). *porcupine*, which has been proposed to be required for production or secretion of *wg* signal, is also non-autonomous (J. Klingensmith, N. Perrimon, and R. Nusse, personal communication; van den Heuvel et al., 1994). In contrast, *arm* (Wieschaus and Riggleman, 1987; Peifer et al., 1991) and *disheveled* (Theisen et al., 1994; J. Klingensmith et al., 1994) are cell autonomous – i.e., mutant cells are not rescued by wild-type neighbors – and thus *arm* and *disheveled* are required on the receiving end of the signal.

To position genes more precisely within the *wg* signaling pathway, Siegfried et al. made use of the mutation *zeste white 3 (zw3)*. *zw3* mutants have a phenotype opposite to that of *wg*, such that all cells in *zw3* mutant embryos adopt a posterior fate and secrete naked cuticle. This allows one to use double mutant analysis to order genes in the *wg* signaling pathway. In a *zw3; wg* double mutant, all cells make naked cuticle, similar to a *zw3* single mutant (Siegfried et al., 1992). This suggests that, at least in a formal genetic sense, *zw3* operates downstream of *wg* in the signaling pathway. By similar criteria, Siegfried et al. (1994) have positioned *disheveled* and *porcupine* upstream of *zw3* , and *arm* downstream of it (Fig. 2C). We have obtained similar results with *zw3* and *arm* (Peifer et al., 1994). Noordemeer et al. (1994) have used a different strategy utilizing a *wg* gene under inducible control to obtain results entirely consistent with these epistasis tests. Together, these results suggest a tentative pathway for transmission of information between cells. It must be remembered, however, that these results cannot be translated directly into a biochemical pathway. To position these genes in a biochemical pathway and to uncover the molecular mechanisms of *wg* signal transduction, information is required as to the nature of the molecules encoded by these genes. As one part of this effort, *arm* was cloned and its product analyzed (Riggleman et al., 1989; Riggleman et al., 1990). This effort, however, lead to a surprising conclusion, providing a cell biological role for Armadillo in cell adhesion and suggesting that cell-cell adhesive junctions play a role in transduction of particular cell-cell signals. To understand this connection, we must first review the current state of knowledge about cell-cell junctions in both insects and vertebrates.

THE ROLES OF CELL-CELL JUNCTIONS IN CELL COOPERATION AND COMMUNICATION

To assemble a multicellular animal, individual cells comprising it must both communicate and cooperate to form well organized tissues. One of the most common solutions to the problem of cellular organization is the epithelial sheet – a sheet of cells one cell thick, with a well-defined apical and basal surface. In a simplified view, cells have to accomplish three things to form an epithelium. They must: (1) adhere to each other, (2) recognize that they have adhered to each other and thus polarize their membrane and assemble other types of junctions, and (3) coordinate their actions, by coordinating their individual cytoskeletons. A single membrane-associated structure, the adherens junction (zonula adherens or belt desmosome; Fig. 3) is thought to initiate all three aspects of this process. The adherens junction was originally identified by morphological criteria as a distinctive region of the membrane near the lateral-apical interface of epithelial cells; recently its molecular components have begun to be identified (Fig. 4A; reviewed by Magee and Buxton, 1991).

The central organizer of the adherens junction is a transmembrane protein of the cadherin family (Fig. 4A). Members of the large and still-increasing cadherin family are present in different tissues and at different developmental stages (reviewed by Takeichi, 1991). The extracellular domains of these molecules interact homotypically to generate cell-cell adhesion. However, cadherins are not simply molecular glue that sticks cells together. The cadherin intracellular domain organizes a multi-protein complex within the cell (Ozawa et al., 1989; Nagafuchi and Takeichi, 1989). This complex is required for adhesion, and also is thought both to mediate interactions with the actin cytoskeleton, and to transmit a signal into the cell upon adhesion. The signal resulting from cadherin interaction is thought to regulate subsequent events such as cell polarization and formation of tight and gap junctions (Gumbiner et al., 1988; Wollner et al., 1992).

The cytoplasmic proteins forming a complex with the cadherin intracellular domain have also been identified (Fig. 4A). Three proteins form the core of this complex, and can be co-purified with cadherins. These proteins, originally identified as proteins that co-immunoprecipitate with E-cadherin, were given the names α-, β-, and γ-catenin (Ozawa et al., 1989). The genes encoding α- and β-catenin have been identified (Nagafuchi et al., 1991; Herrenknecht et al., 1991; McCrea et al., 1991). The protein band identified by one-dimensional SDS-PAGE as γ-catenin appears to actually be composed of two different protein species (Piepenhagen and Nelson, 1993); one of these is plakoglobin (Peifer et al., 1992; Knudsen and Wheelock, 1992), a protein previously identified as a component of both adherens junctions and desmosomes (Cowin et al., 1986). Other proteins are less tightly associated with the adherens junction (Tsukita and Tsukita, 1989); less is known about these proteins though individual components have begun to be characterized (Tsukita et al., 1989a,b; Nelson et al., 1990; Nagafuchi et al., 1991; Itoh et al., 1993).

The molecular components of the other major type of cell-cell adhesive junction, the desmosome, have also begun to be identified (reviewed by Magee and Buxton, 1991). Desmosomes are found in a more restricted set of cell types, and are dispersed along the lateral boundaries of cells. These junctions anchor the intermediate filament cytoskeleton.

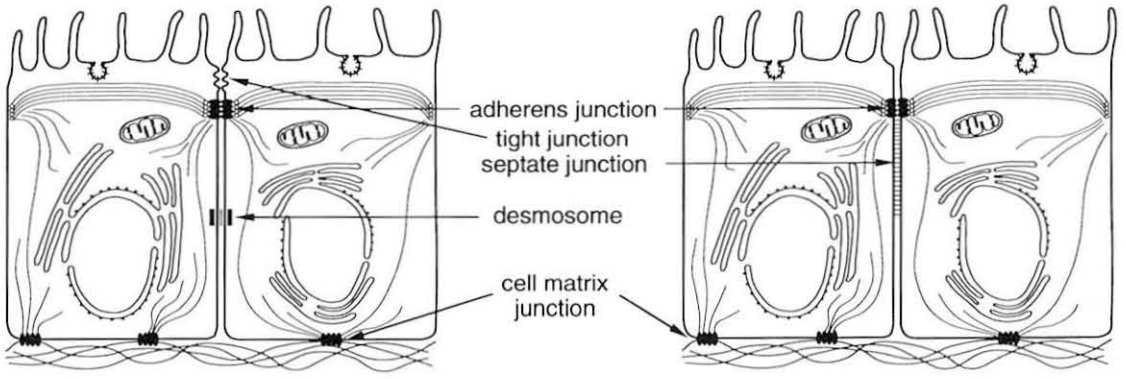


Fig. 3. Cell-cell and cell-matrix junctions in vertebrate and insect cells. Vertebrate (left) and insect (right) epithelial cells are diagrammed. The apical cell surface is at the top and the basal surface at the bottom. Cell-cell and cell-matrix junctions are presented. Both insect and vertebrate cells have similar adherens junctions at the apical-lateral interface. Vertebrate cells also have desmosomes, located along the lateral surface. There is no evidence for the existence of desmosomes in insects. Vertebrate cells have tight junctions just apical to the adherens junctions. The septate junction may play an analogous role in insect cells, though it is found basal to the adherens junction. Both insect and vertebrate cells share similar integrin-containing junctions that mediate contact between the cell and the extracellular matrix.

Desmosomes are organized around transmembrane glycoproteins of the desmoglein and desmocollin families. These proteins are related to the traditional cadherins, forming another branch of the cadherin superfamily. Like traditional cadherins, desmogleins and desmocollins also organize a complex of proteins around their cytoplasmic domains. This complex of cytoplasmic proteins includes plakoglobin, the only known common component of both desmosomes and adherens junctions, and desmoplakin, which appears to be involved in anchoring intermediate filaments.

The cell biology of adhesive junctions and *wg* signaling are connected via the Armadillo protein, which is related in sequence to both β-catenin (McCrea et al., 1991) and plakoglobin (Peifer and Wieschaus, 1990). The similarity between these molecules is quite striking; Armadillo is 71% identical to β-catenin and 63% identical to plakoglobin at the amino acid level. While this degree of identity suggests a similar biochemical role for all three proteins, it does not demonstrate that Armadillo plays a similar cellular role as either of its vertebrate homologs. For example, despite their sequence similarity, β-catenin is a component solely of adherens junctions while plakoglobin is also found in desmosomes – this underscores the danger of assuming too much from sequence similarity. We thus set out to determine whether Armadillo plays a role in an adherens junction complex in *Drosophila*.

Electron microscopy had revealed that epithelial cells in the *Drosophila* embryo and imaginal discs have structures morphologically similar to vertebrate adherens junctions positioned at the lateral-apical cell interfaces (Poodry and Schneiderman, 1970; Eichenberger-Glinz, 1979). Armadillo is enriched in the vicinity of the plasma membrane, and in some cells its localization is polarized, with an enrichment near the apical surface (Riggleman, 1989; Peifer and Wieschaus, 1990). We extended this analysis by examining two simple epithelia of *Drosophila*, the developing embryonic gut (Peifer, 1993), and the somatic follicle cells of the ovary (Peifer et al., 1993), to determine whether Armadillo co-localizes with adherens-like junctions. To do so, we made use of a fixation procedure developed for examination of the cytoskeleton, which washes away much of what we assume is the more loosely bound Armadillo, allowing visualization of the most tightly bound protein. When this is done, Armadillo is dramatically enriched in the same region of the cell in which we found adherens-type junctions (Fig. 4B).

This data is consistent with a role for Armadillo in adherens junctions. To learn more about the junctional components, we examined whether Armadillo existed as part of a multi-protein complex (Peifer, 1993). Most of the Armadillo in the cell is part of a larger complex that includes the *Drosophila* homolog of α-catenin, Armadillo (the β-catenin homolog), and a 150×10^3 M_r glycoprotein that we suspected would be a cadherin homolog, by analogy to the vertebrate adherens junction (Peifer, 1993; Fig. 4A). The Takeichi lab has cloned the gene encoding the 150×10^3 M_r glycoprotein and confirmed that it is related to vertebrate cadherins (M. Takeichi, personal communication). An identical complex of proteins was detected by Oda et al. (1993) when examining proteins associated with *Drosophila* α-catenin. Together, these experiments demonstrate that *Drosophila* and vertebrates have adherens junctions composed of essentially identical proteins.

In the vertebrate system, elegant experiments in tissue culture have demonstrated that adherens junction components have at least some of the properties consistent with the model presented above. Making use of cell lines lacking cadherins, these workers demonstrated that cadherin-negative cells do not exhibit Ca^{2+}-dependent adhesion, but that this property can be conveyed by transfection of the cells with a cadherin gene (Nagafuchi et al., 1987). Transfected cells will also assemble multi-protein junctional complexes, which will then connect to the cytoskeleton (Ozawa et al., 1989; Nagafuchi and Takeichi, 1989). α-catenin-negative cells also lack the ability to adhere to each other; this ability, as well as the ability to form epithelia, can be conferred on these cells by transfection with an α-catenin gene (Hirano et al., 1992). These experiments suggest that adherens junction assembly and its subsequent consequences require at least cadherins and α-catenin.

We extended these experiments to Armadillo, the β-catenin homolog, and have demonstrated the requirement

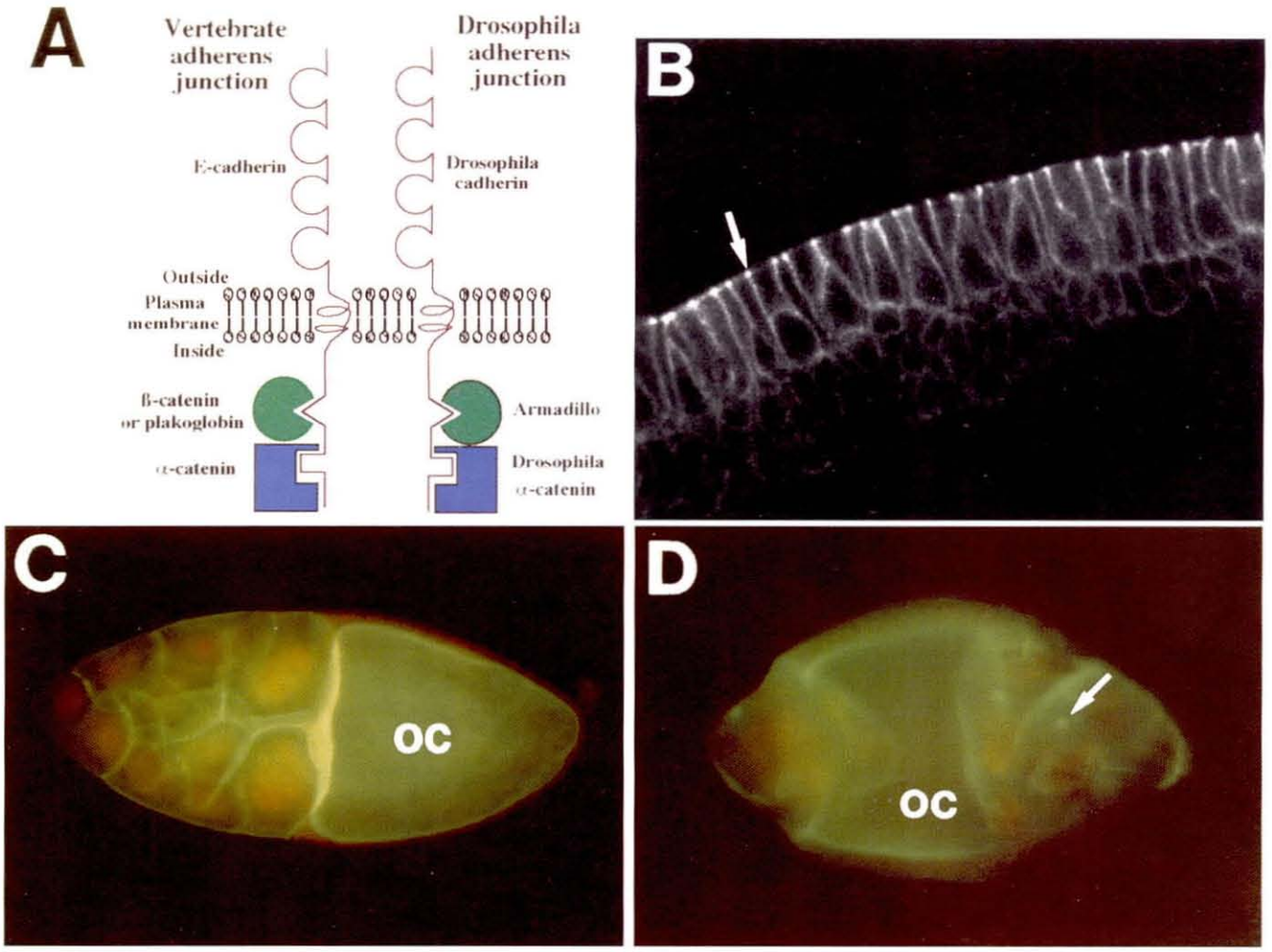


Fig. 4. Vertebrate and insect adherens junctions have similar structures and functions. (A) Molecular structure of the adherens junction in vertebrates and *Drosophila*. In both the junction is organized around a member of the cadherin family of cell adhesion molecules. The cadherin extracellular domain mediates homotypic adhesion with cadherins on neighboring cells, while the intracellular domain organizes the formation of a multi-protein complex. In both vertebrates and *Drosophila* this complex includes Armadillo/β-catenin and α-catenin. (B) Confocal microscope picture of the *Drosophila* embryonic ectoderm stained with anti-Armadillo antibody. Armadillo accumulates all along the lateral face of the cells but is enriched at the apical-lateral interface (arrow), where adherens junctions will form. Wild-type (C) and Armadillo-germline-mutant (D) egg chambers, stained with both propidium iodide to visualize nuclei and with fluorescein-labeled phalloidin to visualize the actin cytoskeleton. Posterior is to the right. In a wild-type egg chamber (C), the germ cells are surrounded by a follicle cell epithelium. The oocyte (oc) is always at the posterior end, and the nurse cells at the anterior. Nurse cells are regular in shape, mononucleate, and have well-defined cortical actin cytoskeletons. Normal cell interactions and the actin cytoskeleton are grossly disrupted when germ cells are mutant for Armadillo (D). The oocyte (oc) can be in the middle or at the anterior end, and nurse cells become irregular in shape. At times the cortical actin cytoskeleton between nurse cells breaks down, leading to the formation of multinucleate germ cells, and leaving behind inclusions of actin (arrow).

for the adherens junction complex during development (Peifer et al., 1993). We used as an experimental system the *Drosophila* ovary, a relatively simple complex of three cell types, follicle cells, nurse cells and an oocyte, that exhibit a highly stereotyped arrangement with respect to each other, presumably regulated by cell-cell interactions (Mahowald and Kambysellis, 1980; Fig. 4C). An epithelial sheet of somatic follicle cells surrounds a set of germ-line cells including a single oocyte and fifteen nurse cells. Within the follicle cell epithelium, the germ cells are arranged with the oocyte at the posterior end and nurse cells at the anterior end, an arrangement presumably maintained by interactions between follicle cells and germ cells. The germ cells have regular shapes and sizes, supported by their cortical actin cytoskeletons.

We compared wild-type ovaries to ovaries in which the germ cells completely lack Armadillo (Peifer et al., 1993). Adherens junctions are predicted to regulate cell adhesion and the anchoring of the actin cytoskeleton. Both properties appear to be disrupted by mutations in Armadillo (Fig. 4D). The normal organization of germ cells within the egg chamber is disrupted, such that the oocyte can be found in the middle of the egg chamber (Fig. 4D) or even at the anterior end. Germ cells become extremely irregular in shape, and at times the cortical actin cytoskeleton seems to break down entirely, resulting in fusion of adjacent nurse

cells and leaving behind cytoplasmic inclusions of actin. The normal migration of a subset of the follicle cells between the nurse cells is often disrupted, as is appropriate packaging of sixteen-cell cysts into egg chambers. Thus, Armadillo appears to be required for cell adhesion and cytoskeletal integrity during normal development, as predicted from theories of adherens junction function.

In addition to mediating cell adhesion and cytoskeletal anchoring, adherens junctions are also thought to regulate transmission of a signal that adhesion has occurred, leading to cell polarization and assembly of gap and tight junctions. The nature of this signal remains mysterious, though in axon outgrowth some downstream events in signal transduction in response to N-cadherin-mediated adhesion have been identified (Schuch et al., 1989; Doherty et al., 1991). Progress has been made in beginning to uncover events that may regulate junctional assembly and disassembly. During development cells both form and leave epithelia. Regulation of this epithelial-mesenchymal transition is important not only for normal development, but also plays a role in cancer metastasis (reviewed in Behrens et al., 1992). One aspect of this transition is the assembly or disassembly of adherens junctions. Using tissue culture models, progress has been made in understanding this event. For example, when one adds to adherent cells the activated tyrosine kinase *v-src*, cells rapidly lose both adherens junctions and cell-cell adhesion (Warren and Nelson, 1987). Several adherens junction proteins are phosphorylated on serine and threonine in normal cells; *v-src* transfection leads to tyrosine-phosphorylation of a subset of these proteins (Matsuyoshi et al., 1992; Behrens et al. 1993; Hamaguchi et al., 1993). There is a strong correlation between loss of adhesion and the state of tyrosine phosphorylation of adherens junction proteins, especially β-catenin (Volberg et al., 1992). This has led to the proposal that β-catenin may serve as the regulatory component of junctional assembly. The ability to be tyrosine phosphorylated has been conserved during evolution, as it is also shared by Armadillo (unpublished data).

Given the similarity between vertebrate and *Drosophila* adherens junctions, it is of interest to know whether other cell-cell junctions are equally conserved. As mentioned above, vertebrates have a parallel set of cell-cell adhesive junctions known as desmosomes, which are for the most part restricted to epithelial cells and which serve to organize intermediate filaments (reviewed in Magee and Buxton, 1991). Desmosomes differ in both morphology and position from adherens junctions; desmosomes have a distinctive multi-layered appearance in the EM and are distributed along the lateral interface. While *Drosophila* cells do have cell-cell junctions along the lateral interface of certain cell types (e.g., germ cells of the ovary; Peifer et al., 1993), these junctions lack the distinctive morphology of desmosomes and are more correctly called spot adherens junctions (Tepass and Hartenstein, 1993; these authors have done an extensive analysis of cell-cell and cell-matrix junctions during *Drosophila* embryogenesis). The existence of cytoplasmic intermediate filaments in *Drosophila* is a matter of disagreement; immunological evidence produced conflicting results, and no one has yet isolated a gene encoding a cytoplasmic intermediate filament. In looking for the housefly Armadillo homolog (Peifer and Wieschaus, 1993), we did not find other related genes that might represent the homolog of vertebrate plakoglobin. Further experimentation will be required to determine whether plakoglobin or desmosomes exist in *Drosophila*.

Another prominent cell-cell junction of vertebrates is the tight junction, which serves to seal epithelial sheets (Fig. 3). This junction is found apical to the adherens junction in vertebrate epithelial cells. Most invertebrate cells lack structures resembling tight junctions; instead they possess an alternate structure known as the septate junction (Fig. 3). Septate junctions are found just basal to adherens junctions in a variety of invertebrate epithelia (Lane, 1991). Some workers have speculated that tight junctions and septate junctions may serve analogous functions in different phyla (Noirot-Timothee and Noirot, 1980), but this question has been difficult to answer in the absence of knowledge of the molecular components of either type of junction. Recently, certain tight junction and septate junction components have been identified (Anderson et al., 1989; Citi et al., 1991; Gumbiner et al., 1991; Zhong et al., 1993; Woods and Bryant, 1991), and the genes encoding some of these products cloned.

Several surprises have emerged from this information. When the Tsukita lab recently cloned a 220×10^3 M_r protein they had originally identified as a component of the cell-cell adherens junction of rat liver (Itoh et al., 1991), they found that it was identical to the vertebrate tight junction protein ZO-1 (Itoh et al., 1993). This has led some to speculate that the distinction between adherens junctions and tight junctions may not be as clear cut as was thought. Certain junctions, such as the cardiac intercalated disc or the adherens junctions that define the bile canaliculi, may have hybrid character as they appear to contain proteins that have been defined as components of both adherens junctions (cadherins, α-catenin) and of tight junctions (ZO-1). Further characterization of the molecular components of both junctional types may help sort this issue out.

The sequence of ZO-1 also revealed another, perhaps even more surprising connection. The ZO-1 protein is related in sequence to the *Drosophila* protein *discs large (dlg*; Woods and Bryant, in press); *dlg* is the progenitor of a family of related vertebrate and insect proteins found in a variety of cell-cell junctions (Woods and Bryant, 1991; Koonin et al., 1992; Bryant and Woods, 1992). In *Drosophila*, *dlg* is found in the septate junctions (Woods and Bryant, 1991), providing the first molecular link between septate and tight junctions. *dlg* also connects the function of these junctions to cell signaling and growth regulation. *dlg* is a tumor suppressor gene, and contains a domain similar to guanylate kinase (Woods and Bryant, 1991). The *Drosophila dishevelled* gene, which is required for both *wg* signaling and for the polarity of hairs and bristles on the body, is also a member of the *dlg* gene family (Theisen et al., 1994). Like adherens junctions, septate junctions may play both structural/architectural roles and also be involved in regulating signaling between cells. The only other identified component of septate junctions in *Drosophila* is a homolog of Band 4.1, a member of the ezrin/radixin/moesin family (R. Fehon, personal communication). Members of this family of proteins are found in a variety of cell-cell and cell-matrix junctions in vertebrate cells (Sato et al., 1992).

A STRUCTURE-FUNCTION STUDY OF Armadillo

Our ultimate goal is an understanding of the biochemical role of Armadillo in both *wg* signaling and cell adhesion. To reach this goal we must integrate our studies of the role of Armadillo at the level of the cell and the organism with information about the precise mechanism by which Armadillo acts to promote these adhesion and signaling. To this end, information about the structure and function of the protein and how these are related becomes essential. Our current working model for Armadillo proposes that it functions within the cell as an "adapter", serving to connect one protein to another, in a manner analogous to that proposed for SH2 and SH3 proteins. Some of the proteins with which Armadillo is likely to interact, such as cadherins and α-catenin, are already known, while others remain to be identified. We thus might expect to find particular domains of Armadillo responsible for interactions with different target proteins.

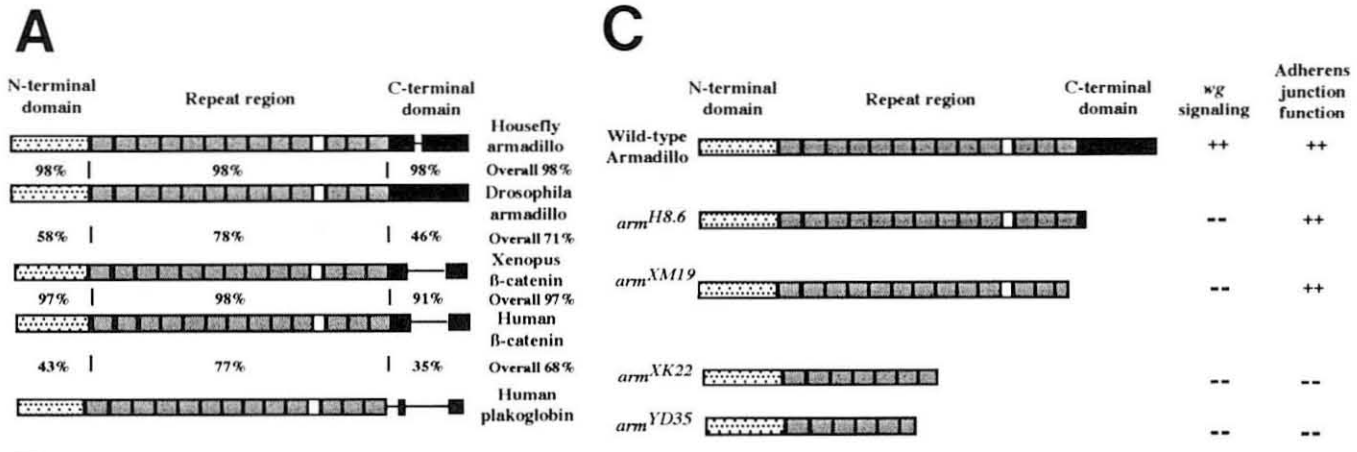
When the sequence of Armadillo became available (Riggleman et al., 1989), one feature of the protein became apparent. Armadillo can be roughly divided into three "domains", defined by the presence of thirteen imperfect 42-amino acid repeats, which make up the central two-thirds of the protein (Fig. 5A,B). The N terminus contains a stretch of acidic residues, while the region C-terminal to the repeats is rich in glycine and proline. These same domains are found in Armadillo's vertebrate homologs, β-catenin (McCrea et al., 1991) and plakoglobin (Peifer and Wieschaus, 1990), but each "domain" is conserved to a different extent (Fig. 5A).

The repeat region is the most highly conserved part of the protein; it is between 75-80% identical between Armadillo, β-catenin, and plakoglobin. The repeats pose an interesting problem of protein evolution. Individual repeats are only 20-30% identical to each other within a single protein (Fig. 5B), probably only retaining sufficient identity to indicate a similar tertiary structure, yet individual repeats are highly conserved among all three proteins. For example, repeat no. 1 of Armadillo, repeat no. 1 of β-catenin, and repeat no. 1 of plakoglobin are 75% identical. The individual repeats appear to have been free to diverge soon after their duplication, yet now are under severe evolutionary constraints. It is possible that individual repeats have independent functions, perhaps mediating different protein-protein interactions, like individual EGF-repeats of the Notch protein mediate interactions with specific ligands (Rebay et al., 1991). The notion of independent and perhaps additive functions of individual repeats is supported by analysis of *arm* mutations. All available *arm* mutations result in protein truncations (Peifer and Wieschaus, 1990). The least truncated protein, encoded by $arm^{H8.6}$, deletes most of the C-terminal domain, while other mutations remove successively more of the protein (Fig. 5C). There is a striking correlation between extent of the deletion and severity of the mutant phenotype. Perhaps the most surprising fact is that the protein encoded by arm^{XK22}, which is only half the length of wild-type protein, retains some small amount of function, supporting an independent and additive role for the repeats.

The N-terminal and C-terminal domains are less well conserved between the three homologs (Fig. 5A). The degree of conservation in the N-terminal domain varies depending on the comparison made. Armadillo and β-catenin are 58% identical in this region, while plakoglobin is only 42-43% identical to the others. This domain may be involved in a function shared by Armadillo and β-catenin, but not by plakoglobin, such as interaction with α-catenin. The C-terminal domain is even less highly conserved; in this domain substantial differences in length are seen among the three proteins with Armadillo the longest and plakoglobin the shortest. Much of the difference is due to the absence of most of the glycine-rich region in β-catenin and plakoglobin. At least part of this glycine-rich stretch is likely to be non-essential, since 20 amino acids of it are missing in housefly Armadillo (Peifer and Wieschaus, 1993). The extreme C terminus is reasonably well conserved, sharing 62% identity over the last 16 amino acids between Armadillo and β-catenin. It is worth noting that within a protein family (e.g., *Drosophila* vs. housefly Armadillo or *Xenopus* vs. human β-catenin; Fig. 5A), all three domains are relatively highly conserved. This suggests that less conserved domains, like the C-terminal region, may play different roles in Armadillo and plakoglobin, but that now these domains may be important for particular plakoglobin-specific or Armadillo-specific protein-protein interactions, leading to their conservation during recent evolution.

Given the dual functions of Armadillo in both *wg* signaling and adherens junctions, one might suspect that particular domains of the protein might be primarily involved in one or the other of these functions. There is evidence from the available mutations that this may be the case. All of the available mutations severely reduce the ability of the protein to participate in *wg* signaling, both in the embryo and during adult pattern formation (Peifer and Wieschaus, 1990; Peifer et al., 1991). Different mutations vary, however, in their effect on Armadillo's junctional function, as assayed during oogenesis (Peifer et al., 1993). Truncated proteins encoded by $arm^{H8.6}$ or arm^{XM19}, which remove the C-terminal domain but leave the repeat region and N-terminal domain intact, are sufficient to fulfill Armadillo's role in oogenesis (Peifr et al., 1993), and clones of cells mutant for these alleles survive in regions of the adult epidermis that do not require *wg* signaling (Peifer et al., 1991). In contrast, truncated proteins encoded by arm^{YD35} or arm^{XK22}, that remove substantial portions of the repeat region, disrupt oogenesis (Peifer et al., 1993), and these alleles are cell lethal in all parts of the adult epidermis (Peifer et al., 1991).

This suggests that the C-terminal domain may not be required for Armadillo's role in the adherens junction, but that it does play an important role in *wg* signaling. This could involve a role in transduction of *wg* signal, perhaps interacting with a hypothetical effector. This suggestion is further supported by our observations concerning a potential role for Armadillo in the nervous system. Armadillo is expressed prominently in axons, where it is presumably playing an adhesive role in axon guidance or axon fasciculation. We have recently determined that an alternative isoform of Armadillo is expressed in the nervous system; this isoform is produced by alternative splicing and lacks the C-terminal domain of the protein entirely (H. Harkins, J.L., and M.P., unpublished data). In the CNS, the N-terminal



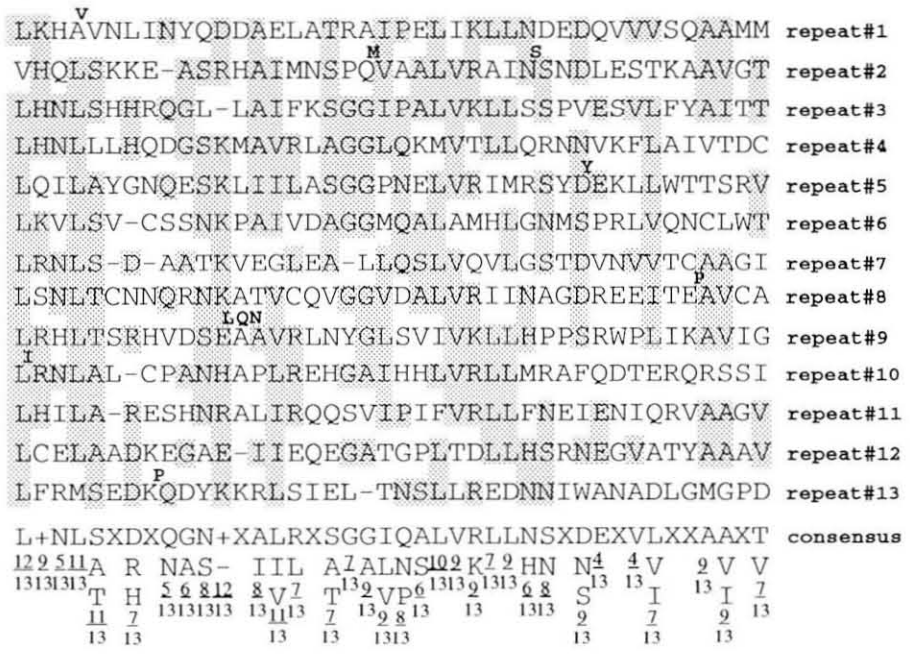


Fig. 5. Structure/function studies of Armadillo protein. (A) The structure of Armadillo, β-catenin, and plakoglobin are compared. Amino acid sequence identities between different family members are indicated. All share a highly conserved region of thirteen imperfect 42-amino acid repeats. The N-terminal and C-terminal regions are less well conserved between Armadillo, β-catenin, and plakoglobin, but are highly conserved within a given type of protein. (B) Sequence comparison of the repeats of Armadillo, using the one-letter amino acid code. Repeats are not very well conserved with respect to each other, with only 20-30% amino acid identity. A consensus is indicated at the bottom; the number of repeats that match the consensus at particular positions are indicated. Matches to the consensus are shaded. An X is placed where no consensus can be found. Small letters above individual repeats are amino acid insertions at that point; a dash indicates a gap that has been introduced. (C) The C-terminal domain of Armadillo is critical for *wingless* signaling and less important for adherens junction function. Four different mutant proteins, all the result of truncations are diagrammed. Their effects on *wingless* signaling and adherens junction function are displayed. Proteins lacking the C-terminal domain (*arm*$^{H8.6}$ and *arm*^{XM19}) retain adherens junction function but have lost function in *wingless* signaling. Proteins missing substantial numbers of repeats have also lost adherens junction function.

domain and repeat region apparently suffice for full function. To explore the role of individual domains in greater detail, we are currently using in vitro mutagenesis to alter specific parts of the protein (S.O. and M.P., unpublished data), and we are expressing individual domains of the protein to assay their role in particular protein-protein interactions (L.-M. P. and M.P., unpublished data).

INTEGRATING Armadillo's ROLES IN CELL ADHESION AND IN CELL-CELL SIGNALING

As outlined above, we have obtained strong experimental support for the idea that Armadillo is required for cells to properly interpret *wg* signal (Wieschaus and Riggleman, 1987; Peifer et al., 1991). We have also demonstrated that the sequence similarity between Armadillo and β-catenin (McCrea et al., 1991) reflects a role for both proteins as components of the adherens junction (Peifer, 1993), and have shown that Armadillo is required for cell adhesion and integrity of the actin cytoskeleton (Peifer et al., 1993). However, this still leaves one key piece of the puzzle to find, the connection between adherens junction function and *wg* signaling.

Two different explanations are possible. The first is an indirect one. A variety of cell-cell signaling molecules of both vertebrates and insects are localized to the adherens junction (Maher and Pasquale, 1988; Takata and Singer, 1988; Tsukita et al., 1991; Fehon et al., 1991; Tomlinson et al., 1987; Bennett and Hoffmann, 1992). In retrospect, it not surprising that this molecular machinery would be assembled in a particular region of the cell surface, allowing

efficient interaction between ligand and receptor, rather than dispersing signals and receptors all over the surface of the cell. The adherens junction appears to be one of the hotspots for cell-cell signaling. One possible explanation for the role of Armadillo, and by extension, adherens junctions, in the transmission of *wg* signal is that the putative Wingless receptor is normally localized to these junctions. In an *arm* mutant, the resulting disruption of junctional function would lead to receptor mis-localization and the failure of *wg* signaling.

Alternatively, Armadillo (and by extension adherens junctions) might play an unexpected direct role in *wg* signal transduction. Several observations support a direct role for Armadillo in *wg* signaling. First, mutations in Armadillo specifically disrupt *wg* signaling, while signaling by Notch and other cell-cell signaling molecules localized to the adherens junction is unaffected. Second, *wg* signaling is especially sensitive to relatively small reductions in *arm* function that fail to disrupt Armadillo's role in cell adhesion during oogenesis (see above). Third, when one examines zygotic *arm* mutants in which *wg* signaling is disrupted, junctional structure and cell adhesion appear roughly normal until quite late in development (D. Sweeton, M. P., and E. Wieschaus, unpublished data). These experiments, while suggestive, do not rule out an indirect role for Armadillo in *wg* signaling. To do so, we and others have used genetic epistasis analysis to order *arm* and other *wg*-class genes in the *wg* signaling pathway (Siegfried et al., 1992; Peifer et al., 1994; Siegfried et al., 1994). This analysis clearly places *arm* downstream of *zw3* and by extension downstream of *wg*, providing strong evidence that *arm* and thus adherens junctions are directly involved in transduction of *wg* signal.

We have begun to investigate the mechanism by which Armadillo transduces *wg* signal. While *arm* mRNA is uniformly distributed throughout the embryonic epidermis (Riggleman et al., 1989), Armadillo protein accumulation is segmentally striped (Riggleman et al., 1990). Armadillo stripes are a direct visualization of *wg* signal transduction. The stripes roughly coincide with the graded stripes of Wingless (Riggleman et al., 1990; Gonzalez et al., 1991; Peifer et al., 1994), and the formation of stripes does not occur in a *wg* mutant, leaving all cells with the distribution of Armadillo seen in wild-type interstripe cells (Riggleman et al., 1990; Peifer et al., 1994). To understand the role of stripe formation in *wg* signaling, we have examined the mechanism by which stripes are generated. We have found that *wg* signal triggers a dramatic increase in the accumulation of cytoplasmic Armadillo protein (Peifer et al., 1994). Further, we found that mutations in Zeste-white 3 kinase result in accumulation of cytoplasmic Armadillo, even in cells that do not receive *wg* signal (Peifer et al., 1994), consistent with the position of *arm* downstream of *zw3* determined by epistasis analysis.

We believe it likely that the cytoplasmic Armadillo that accumulates in response to *wg* then interacts with a cellular effector to transduce the signal. Fig. 6 presents two possible models of this process. In one model, *wg* signal and Zeste-white 3 kinase regulate the stability of cytoplasmic Armadillo; in this view the increase in cytoplasmic Armadillo drives assembly of a larger number of cadherin-catenin complexes, altering cell adhesion, and by this mechanism may alter the interaction between constitutive ligands and receptors that then transmit a further signal. In the other model, *wg* signal and Zeste-white 3 kinase regulate the assembly state of adherens junctions, and the putative effector is a cytoplasmic protein, such as a kinase, which acts to further transmit *wg* signal to its ultimate targets. These models are purely speculative, and components of the two models are interchangeable. For example, one might imagine that *wg* signal and Zeste-white 3 kinase regulate stability of cytoplasmic Armadillo as in Model 1, but that this cytoplasmic Armadillo then stimulates a cytoplasmic effector as in Model 2. One of our current goals is to fill in the biochemical details of Armadillo's role in signal transduction.

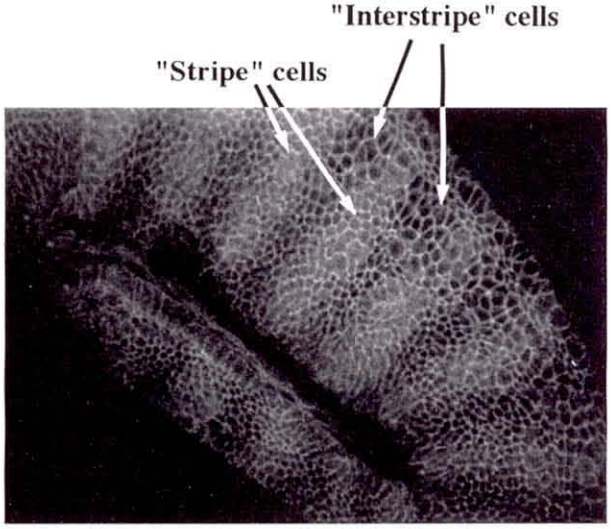


Fig. 6. Armadillo protein stripes. Confocal microscope image of a stage 9 *Drosophila* embryo stained with anti-Armadillo antibody. While *armadillo* RNA is uniformly distributed, Armadillo protein forms segmentally repeated stripes. "Stripe" cells, which have received *wingless* signal, accumulate high levels of cytoplasmic Armadillo, while "interstripe" cells, which do not receive *wingless* signal, have only membrane-associated Armadillo.

One of the most exciting discoveries of the past 10 years of developmental biology is that the same or similar molecules are operating to regulate development in a wide variety of organisms. Perhaps the best known example is that of the homeotic genes, that regulate identity along the anterior-posterior body axis in animals as diverse as nematodes, *Drosophila*, and mammals. The *wg/wnt-1* system provides another example for a conserved regulatory circuit. *wg/wnt-1* genes are conserved in a wide variety of animal phyla (e.g., Kamb et al., 1989), and in insects and mammals are known to regulate cell fate decisions during development (reviewed in Peifer and Bejsovec, 1992; McMahon, 1992). Given this conservation of signal, it is of interest to ask whether the machinery to receive and interpret the signal has also been conserved.

Many of the other molecules involved in *wg* signaling are highly conserved. Most organisms thus far examined have a highly conserved homolog of the *engrailed* gene, which encodes a transcription factor that appears to be one of the

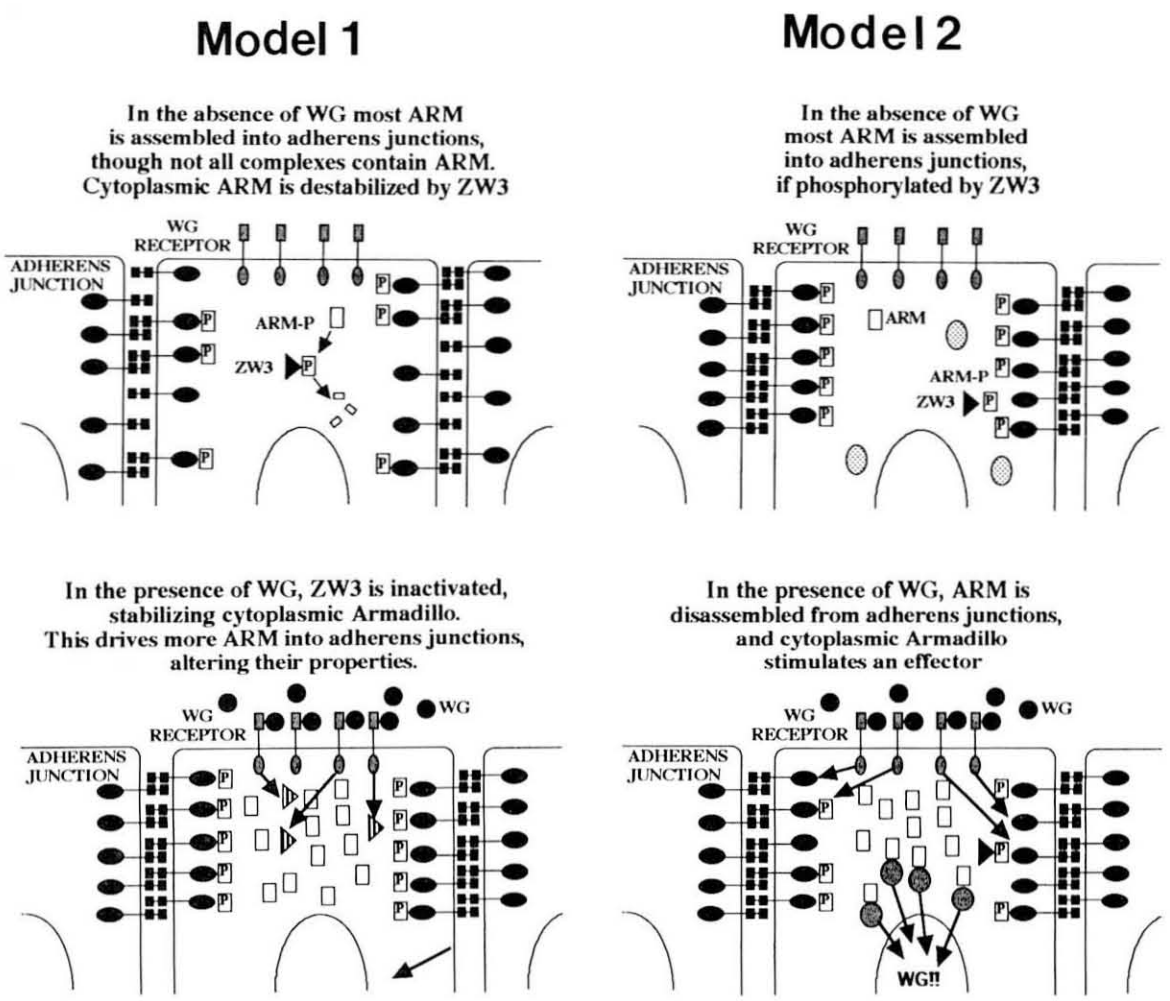


Fig. 7. Two speculative models for how Armadillo may function in *wingless* signal transduction. In Model 1, cytoplasmic Armadillo is normally unstable, due to the action of Zeste-white 3 kinase. *wingless* signal acts to counter this instability, perhaps by inactivating Zeste-white 3 kinase. Levels of cytoplasmic Armadillo rise, driving more Armadillo into the adherens junction complex at the membrane. This alters cell-cell adhesion, and by some mechanism results in further transduction of *wingless* signal. In Model 2, in the absence of *wingless* signal most Armadillo is normally found in the adherens junction complex. Assembly of this complex is promoted by Zeste-white 3 kinase. *wingless* signal acts to destabilize the adherens junction complex, releasing Armadillo into the cytoplasm. This cytoplasmic Armadillo then interacts with a cytoplasmic effector such as a kinase to transduce *wingless* signal.

targets of *wg* signal, and in both the mammalian brain and the *Drosophila* epidermis, cells expressing Wingless are juxtaposed to cells expressing Engrailed (reviewed in Peifer and Bejsovec, 1992). Likewise, Armadillo is 71% identical to β-catenin (McCrea et al., 1991), and Zeste-white 3 is 76% identical to GSK-3β (Siegfried et al., 1992; de Groot et al., 1993). This by itself, however, does not prove that the mammalian homologs are required for *wnt* signaling. The definitive resolution of this question will require that mutations be made in β-catenin and GSK-3β. An intriguing hint has emerged suggesting that *wnt* signaling will operate via the same pathway as that for *wg*. When anti-β-catenin antibody is injected into *Xenopus* embryos, it results in dorsal axis duplication (McCrea et al., 1993) very similar to that produced by *wnt* RNA injection (McMahon and Moon, 1989; Sokol et al., 1991; Smith and Harland, 1991). Further, Bradley et al. (1994) 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 shown that this change alters cell adhesive properties. These results, together with those from *Drosophila*, suggest that Armadillo/β-catenin/plakoglobin and adherens junctions are part of a conserved set of cellular machinery required for transduction of particular cell-cell signals. Our current challenge is to define the biochemical role of Armadillo in the *wg* signal transduction process.

We very grateful to all of our colleagues who shared data before publication and provided valuable insight into its interpretation. We would like to thank especially D. Sweeton and E. Wieschaus, who have helped shape our view of Armadillo's role in development. We would also like to thank A. Bejsovec, N. DuBois, H. Harkins, and M. Valencik who helped clarify the manuscript, and S. Whitfield for talented graphic artistry. Work in our lab is supported by grants from the N.I.H., the Searle Scholars Program, and the March of Dimes.

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