An In Vivo Structure-Function Study of Armadillo, the β-Catenin Homologue, Reveals Both Separate and Overlapping Regions of the Protein Required for Cell Adhesion and for Wingless Signaling

Sandra Orsulic and Mark Peifer

Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-3280

Abstract. Armadillo, the *Drosophila* homologue of vertebrate β-catenin, plays a pivotal role both in Wingless signaling and in assembly of adherens junctions. We performed the first in vivo structure-function study of an adherens junction protein, by generating and examining a series of Armadillo mutants in the context of the entire animal. We tested each mutant by assaying its biological function, its ability to bind proteins that normally associate with Armadillo in adherens junctions, its cellular localization, and its pattern of phosphorylation. We mapped the binding sites for DE-cadherin and α-catenin. Although these bind to Armadillo independently of each other, binding of each is required for the function of adherens junctions. We identified two separate regions of Armadillo critical for Wingless signaling. We demonstrated that endogenous Armadillo accumulates in the nucleus and provide evidence that it may act there in transducing Wingless signal. We found that the Arm repeats, which make up the central two-thirds of Armadillo, differ among themselves in their functional importance in different processes. Finally, we demonstrated that Armadillo's roles in adherens junctions and Wingless signaling are independent. We discuss the potential biochemical role of Armadillo in each process.

ARMADILLO is part of the Wingless signal transduction pathway, responsible for establishing segment polarity of *Drosophila* embryos and adults (Wieschaus et al., 1984; Peifer, 1995). It was thus surprising that its vertebrate relatives β-catenin and plakoglobin are components of cell–cell adherens junctions. Adherens junctions are adhesive junctions located at the apical end of the lateral surfaces of all epithelial cells; they also are present in many nonepithelial cells. They are thought to mediate cell–cell adhesion, to act as sensors of this adhesion, and to anchor the actin cytoskeleton. Adherens junctions are organized around transmembrane cadherin proteins. Extracellular cadherin domains mediate cell–cell adhesion, while intracellular domains organize a multiprotein complex including α- and β-catenin, plakoglobin, and p120 (for review see Kemler, 1993). β-Catenin links cadherins to α-catenin (Aberle et al., 1994; Hülsken et al., 1994; Oyama et al., 1994; Jou et al., 1995). α-Catenin is thought to bind to actin (for review see Kemler, 1993). Catenins play an essential role in regulating cadherin function (for review see Kemler, 1993).

Please address all correspondence to M. Peifer, Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-3280. Tel.: (919) 962-2271. Fax: (919) 962-1625. E-mail: peifer@unc.edu

Given the sequence similarity between Armadillo and β-catenin (71% amino acid identity), it has been confirmed that both have similar biochemical functions. Armadillo is found in *Drosophila* adherens junctions along with α-catenin and cadherin homologues (Peifer, 1993; Oda et al., 1993, 1994). Armadillo function is necessary in adherens junctions of both ovaries (Peifer et al., 1993) and embryos (Cox et al., 1996); in Armadillo's absence cell–cell adhesion, cell polarity, and cytoskeletal integrity are disrupted. *Drosophila* E-cadherin is also required in epithelial cells (Uemura et al., 1996; Tepass et al., 1996). Analogous experiments in mice revealed roles for both E-cadherin and β-catenin during embryogenesis (Larue et al., 1994; Riethmacher et al., 1995; Haegel et al., 1995). Thus, both Armadillo and β-catenin share key roles in adherens junctions, and adherens junction function is critical for embryogenesis.

β-Catenin, like Armadillo, also has a role in signaling. Many signaling molecules related to Wingless exist in vertebrates. These Wnt proteins mediate numerous cell fate choices during embryogenesis (for review see Parr and McMahon, 1994); for example, a Wnt protein plays a key role in setting up the dorsal-ventral axis in frogs. Vertebrate homologues of most known components of the Wingless signal transduction pathway have been identi-

fied. All, including β-catenin, play a role in Wnt signaling (for review see Klymkowsky and Parr, 1995).

Are the roles of Armadillo/β-catenin in adherens junctions and in Wingless/Wnt signaling separable or identical, when both roles are required in a single cell? Three lines of evidence support the idea that Armadillo/β-catenin plays independent roles in these two processes, involving separate biochemical complexes (for review see Peifer, 1995). First, in cells responding to Wingless, there are two separate pools of Armadillo: (*1*) adherens junction–associated Armadillo, which is found in virtually all cells, and the accumulation of which is unaltered by Wingless, and (*2*) cytosolic Armadillo that accumulates in response to Wingless via an effect on Armadillo protein stability; this pool of intracellular Armadillo is only found in cells that receive Wingless signal, and is thought to mediate its transduction. Second, certain *armadillo (arm)*[1] mutations like $arm^{H8.6}$ (Peifer et al., 1994*b*) specifically affect Wingless signal transduction but not adherens junction function. Third, cadherin overexpression in *Xenopus* suppresses β-catenin's role in Wnt signaling (Heasman et al., 1994), while in *Drosophila*, reduction in DE-cadherin levels enhances Armadillo's role in Wingless signaling (Cox et al., 1996). Together, these data suggest that Armadillo/β-catenin's roles in adherens junctions and Wingless/Wnt signaling are competitive rather than identical.

Catenins may also be involved in other cell signaling processes outside of adherens junctions. At least one other catenin-containing protein complex exists in the cell. This complex is assembled around the tumor suppressor gene product APC, which associates in a cadherin-independent fashion with α- and β-catenin (Su et al., 1993; Rubinfeld et al., 1993; Hülsken et al., 1994; Rubinfeld et al., 1995). The function of the APC-catenin complex remains a mystery, though circumstantial evidence suggests that APC may regulate β-catenin's role in Wingless/Wnt signaling (Munemitsu et al., 1995; Rubinfeld et al., 1996) and perhaps in other signaling pathways.

Catenins are expressed in most cells at high and constant levels, so regulation of their function is likely to be posttranscriptional. One possible regulatory mechanism is phosphorylation. Both Armadillo (Peifer et al., 1994*a*) and β-catenin (Hamaguchi et al., 1993) are phosphorylated on Ser/Thr and Tyr. There are correlations between phosphorylation and function both in Wingless signaling (Peifer et al., 1994*a*) and in adherens junctions; Tyr phosphorylation of β-catenin correlates with loss of cell adhesion and transformation (for review see Hinck et al., 1994). Certain tyrosine kinases accumulate at adherens junctions (for review see Kirkpatrick and Peifer, 1995); the EGF receptor, a transmembrane tyrosine kinase, interacts directly with β-catenin (Hoschuetzky et al., 1994). Tyrosine kinases may phosphorylate and thus regulate junctional components, and also may be regulated in turn by cell adhesion, perhaps participating in the contact inhibition signal.

To understand the regulation and function of Armadillo and its homologue β-catenin, we must extend the observations made in tissue culture by examining protein function in vivo. Here we report the first detailed structure-function analysis of an adherens junction component in vivo, dissecting the role of *Drosophila* Armadillo in cell adhesion and Wingless signaling. We generated nine different mutations which together affect most regions of Armadillo protein. We then systematically assayed the biochemical, cell biological, and genetic properties of these mutant proteins in order to assign particular functions to different portions of Armadillo protein. Together, these data allowed us to begin to define the biochemical roles and mechanisms by which Armadillo promotes cell adhesion and cell signaling.

Materials and Methods

armadillo Mutant Constructs, P Element Transformation, and Fly Stocks

An *armadillo* minigene was used for the generation of mutations by in vitro mutagenesis (Table I, *left*; details available upon request). Mutants were epitope-tagged with a human c-*myc* epitope (recognized by mAb 9E-10; Evan et al., 1985). One copy of a double-stranded oligonucleotide encoding the 11–amino acid epitope plus two amino acids encoded by flanking NheI sites, 5'CTAGCGAGCAGAAACTGATCTCTGAAGAAGACCTGAACG 3', was inserted into the NheI site (amino acid [a.a.] 784) in the Gly/Pro-rich region close to the COOH terminus of Armadillo. S2 has only the c-*myc* tag inserted; it was used to create all other alleles, which were all (except S8) also epitope-tagged. Deletions and point mutations were introduced into a BamHI subclone carrying the *armadillo* coding region using an in vitro mutagenesis kit (United States Biochem. Corp., Cleveland, OH) as recommended by the manufacturer. Oligonucleotides with desired mismatches were 20–40 nucleotides long. The mutations were S4 (a.a. 63 E→A and a.a. 64 E→A); S5 (a.a. 309-351 deleted); S6 (a.a. 170 D→A, 171 E→A, 172 D→A); S8 (stop codon after a.a. 757); S11 (a.a. 226-392 deleted); S12 (a.a. 549-606 deleted); S14 (a.a. 101-139 deleted); and S15 (a.a. 432-475 deleted). Mutant BamHI fragments were introduced into the BamHI site of a modified version of the P element vector pW8 (Klemenz et al., 1987). The vector had been altered so that most polylinker sites were deleted and so that it contained the *armadillo* promoter and 3' trailer (Riggleman et al., 1989). Flies were injected as in Spradling (1986). Several homozygous lines were established for each construct, and mutant protein expression was tested on Western blots. The *y w* stock (arm^{+}) was used for microinjection and as a control. The embryonic and germline phenotypes of arm^{YD35}, $arm^{H8.6}$, and arm^{XP33} are described in Peifer and Wieschaus (1990) and Peifer et al. (1993). Stocks for producing germline clones are described in Chou and Perrimon (1992).

Genetic Tests of Function and Cuticle Preparation

We carried out four genetic tests of each mutation (tests were done at 25°). Each test was done with at least two independent lines of each mutant, which were previously confirmed to express at least wild-type levels of mutant protein by immunoblotting (SX refers to mutants S2-S15). First, we tested the ability to rescue the zygotic lethality of a null mutation, arm^{YD35}, by crossing $arm^{YD35}/FM7$ females to SX homozygous males. F1 progeny were examined in two ways: (*1*) Eggs were collected for 24 h and hatch rates determined. Cuticle preparations were made of both hatched larvae and unhatched embryos. (*2*) Adult progeny were scored for viability. Second and third, we tested the ability of each mutation to rescue animals with a maternal and zygotic contribution composed entirely of arm^{XP33} or $arm^{H8.6}$ mutant protein. Germline clones of arm^{XP33} or $arm^{H8.6}$ were generated as in Peifer et al. (1993) and females carrying such germline clones were mated to SX homozygous males. Progeny were examined as described above. Fourth, we tested whether mutant proteins could replace both the maternal and zygotic Armadillo contribution, by constructing the stock arm^{YD35} *FRT101/FM7; SX/+*, and crossing females of this genotype to males of the genotype ovo^{D1} *FRT101/Y; hs-flp-F38/hs flp-F38.* Germline clones were generated in progeny as in Peifer et al. (1993). Among the progeny are females with germlines homozygous for arm^{YD35} which also have a copy of the mutant SX gene. We examined oogenesis in these females and examined their progeny as described above. Cuticles were prepared as in Wieschaus and Nüsslein-Volhard (1986).

1. *Abbreviation used in this paper: arm, armadillo.*

Antibodies, Immunoprecipitation, and Immunoblotting

We used c-*myc* mAb (purified from 9E10 cell supernatant; Evan et al., 1985), polyclonal anti-Armadillo antibody (Ab) N2 (Riggleman et al., 1990), anti-Armadillo mAb 7A1 (Peifer et al., 1994*b*), anti-*D*-α-catenin Ab and anti-DE-cadherin DCAD1 Ab (Oda et al., 1993, 1994). Ab concentrations used were (*1*) for immunofluorescence anti-c-*myc* and anti-Armadillo N2 at 1:200, and (*2*) for immunoblotting Arm 7A1 (1:7500), 9E10 cell supernatant (undiluted), α-catenin (1:20), and DCAD1 (1:100). Secondary Abs were preadsorbed overnight with fixed embryos or ovaries. Immunoprecipitation and immunoblotting were as in Peifer (1993). For immunoblotting, proteins were detected by ECL (Amersham Corp., Arlington Heights, IL) or by using alkaline phosphatase–coupled secondary Ab and the detection reagents NBT and BCIP (Promega, Madison, WI), both as recommended by the manufacturer.

Whole-Mount Immunocytochemistry

We used a modification of the method of Blair (1993). Eggs were collected, dechorionated in 50% bleach, and fixed in a glass vial in 1:1 heptane:2× "Brower fix" (4% formaldehyde, 2% NP-40 in PEM) overnight on a nutator at 4°C. Fixative was removed and fresh heptane added. Embryos were transferred to a flat plastic surface and picked up on double stick tape after the heptane evaporated. The tape was inverted and the embryos covered with a drop of water and devitellinized with a sharp razor blade. Embryos were postfixed in 2% formaldehyde, 1% NP-40 in PEM for 3 h on a nutator at 4°C, washed for 1 h in PBS with 0.3% Triton X-100 (PBST) at 4°C, and incubated overnight at 4°C in 1:200 c-*myc* Ab: PBST or the same concentration of Arm N2 polyclonal Ab. After 1 h of washing, embryos were incubated in rhodamine-labeled secondary Ab (Boehringer Mannheim Corp., Indianapolis, IN; previously preadsorbed against embryos or ovaries overnight at 4°C) at 1:500 for 2–3 h. Embryos were washed for 1 h and mounted in Aquapolymount (Polysciences, Inc., Warrington, PA). Ovaries were dissected in Ringer's solution and fixed in 2% formaldehyde, 1% NP-40 in PEM for 30 min at room temperature. They were then treated as the embryos.

Results

Design and Generation of Mutant armadillo Alleles

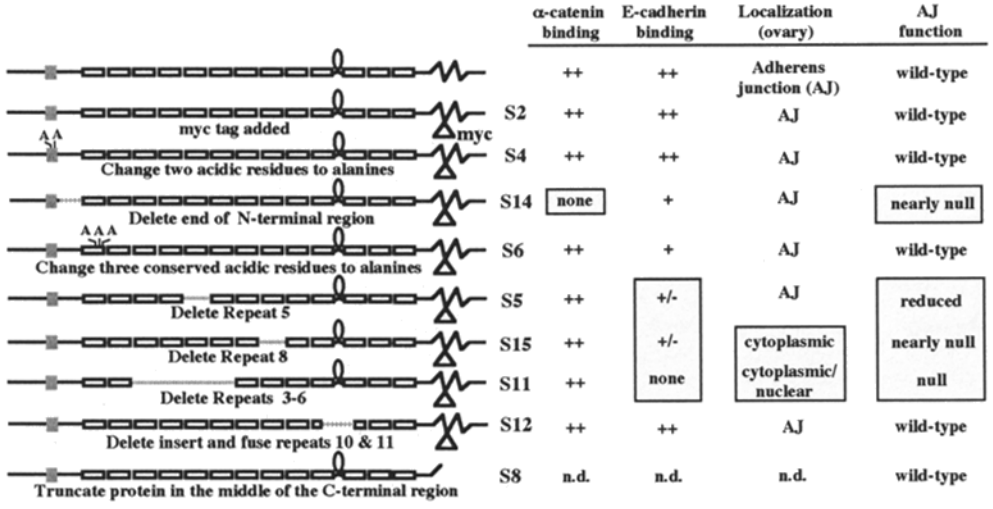
Armadillo protein can be divided into three regions (Table I, left): an NH_2 terminus with a block of acidic residues, a Gly/Pro rich COOH terminus, and the central two-thirds of the protein, consisting of 13 imperfect 42 amino acid Arm repeats, a conserved protein–protein interaction motif found in a variety of otherwise unrelated proteins (Riggleman et al., 1989; Peifer et al., 1994*c*). Between repeats 10 and 11 are 29 amino acids unrelated to Arm repeats. Armadillo's vertebrate homologues have the same overall structure: β-catenin, an adherens junction protein, has 71% amino acid identity to Armadillo, while plakoglobin, found in both desmosomes and adherens junctions, is 63% identical. The level of conservation of different domains varies. While different Arm repeats of Armadillo are only 20–30% identical, corresponding repeats of Armadillo, β-catenin, and plakoglobin are 75–80% identical—all repeats are highly conserved. The NH_2 terminus is somewhat more variable. The COOH terminus is the least conserved region both in sequence and in length.

We generated mutations in vitro spanning much of the protein; some were deletions and others were point mutations altering conserved amino acids (Table I, *left*). The deletions in the repeat region were designed to delete entire repeats, fusing analogous amino acids in adjacent repeats. We reintroduced mutant genes into flies by P element mediated transformation. To distinguish mutated from wild-type Armadillo, we tagged all mutated constructs (except S8) with a human c-*myc* epitope inserted in the Gly/Pro-rich region of the COOH terminus (Table I, *left*). Part of this region is missing in housefly Armadillo (Peifer and Wieschaus, 1993), and thus we suspected it was nonessential. The tag does not interfere with Armadillo function, as the tagged but otherwise wild-type *arm* rescues an embryonic lethal null *arm* allele to adult viability and fertility (see below).

α-Catenin Binds to Armadillo at the Junction of the NH_2 Terminus and the Repeats

The most prominent proteins in adherens junctions are cadherins and their associated catenins (for review see

Table I. Effects of Mutations on DE-cadherin or α-Catenin Binding, and on Adherens Junction Localization and Function



Schematic representation of wild-type and mutant Armadillo protein. The shadowed box is an acidic region in the N-terminus. The open boxes represent 13 Arm repeats with an insertion between repeats 10 and 11. The zig-zagged region is a Gly/Pro-rich region in the C-terminal domain. S2 differs from wild-type only in the insertion of a 13-amino acid c-myc tag. Mutations in the other proteins are indicated (mutations are described in detail in the Methods).
n.d. - not determined.

Kemler, 1993). α-Catenin does not bind directly to cadherin, instead, β-catenin links cadherin and α-catenin (Aberle et al., 1994; Hülsken et al., 1994; Oyama et al., 1994; Jou et al., 1995). By analogy, Armadillo is predicted to link DE-cadherin and α-catenin. Indeed, antibodies to Armadillo coimmunoprecipitate these two proteins (Peifer, 1993; Oda et al., 1993, 1994). We identified regions of Armadillo responsible for interaction with α-catenin and DE-cadherin in vivo by a coimmunoprecipitation assay (Fig. 1; Table I). If a mutation prevents mutant Armadillo from interacting with a particular partner, that partner should no longer coimmunoprecipitate. We used the c-*myc* epitope to specifically immunoprecipitate mutant rather than wild-type Armadillo. The Armadillo mutant S14, lacking 39 amino acids of the NH_2-terminal domain, is the only mutant that does not interact with α-catenin in our assay (Fig. 1 *c*). No other mutation, including those resulting in large deletions of other parts of Armadillo, abolishes α-catenin binding (Fig. 1 *c*).

The Central Arm Repeats Are Required for Both Cadherin Binding and Localization to Adherens Junction

We localized the in vivo DE-cadherin–binding site to the central Arm repeats (Fig. 1 *b*, Table I). All three mutants with deletions in central Arm repeats (S5, S15, and S11) show reduced DE-cadherin binding in a coimmunoprecipitation assay, while mutations in the more peripheral Arm repeats do not affect binding. Central repeat mutants differ in the strength of their DE-cadherin binding; S15 and S5, each lacking a single central repeat, bind very weakly in comparison to the S2 control, while S11, lacking several central repeats, does not bind to DE-cadherin at all. S15 and S11 also show reduced localization to adherens junctions in ovarian follicle cells, as visualized by immunofluorescence (Fig. 2; Table I), suggesting that association with DE-cadherin mediates assembly of Armadillo into adherens junctions. The localization of mutant proteins in adherens junctions is consistent with the strength of binding to DE-cadherin in the biochemical assay (Figs. 1 and 2). The amount of Armadillo accumulating in adherens junctions is inversely related to the amount in the cytoplasm; wild-type protein accumulates almost exclusively in junctions (Fig. 2) while S11 is found almost entirely in the cytoplasm (Fig. 2 *a*); it is also perinuclear in late-stage follicle cells (Fig. 2 *b*). Not all mutations in the repeats have strong effects on DE-cadherin binding. S12, with repeats 10 and 11 fused, binds normally to DE-cadherin (Fig. 1 *b*). S6, which has a triple-point mutation in repeat 1, shows only slightly reduced binding (Fig. 1 *b*), and essentially wild-type localization to adherens junctions (data not shown). Mutation of the α-catenin–binding site does not block DE-cadherin binding; S14 binds DE-cadherin nearly as well as the S2 control (Fig. 1 *b*). Removal of the α-catenin binding site also does not prevent assembly into adherens junctions (Fig. 2).

Wild-Type Armadillo and Certain Mutant Proteins Accumulate in Nuclei in Response to Wingless

We also analyzed the accumulation of mutant proteins in embryos, where Armadillo is simultaneously required in adherens junctions and Wingless signaling. Wild-type Armadillo accumulates at the plasma membrane of all embryonic ectodermal cells, as part of the adherens junction, but in cells that do not receive Wingless, little Armadillo accumulates outside of junctions. In cells receiving Wingless signal, however, wild-type Armadillo also accumulates inside cells, both in the cytoplasm and the nucleus (Fig. 3; Peifer et al., 1994*b*). Wingless signal triggers this increase in intracellular Armadillo by increasing its stability (van Leeuwen et al., 1994).

We focused on the accumulation of mutant Armadillo

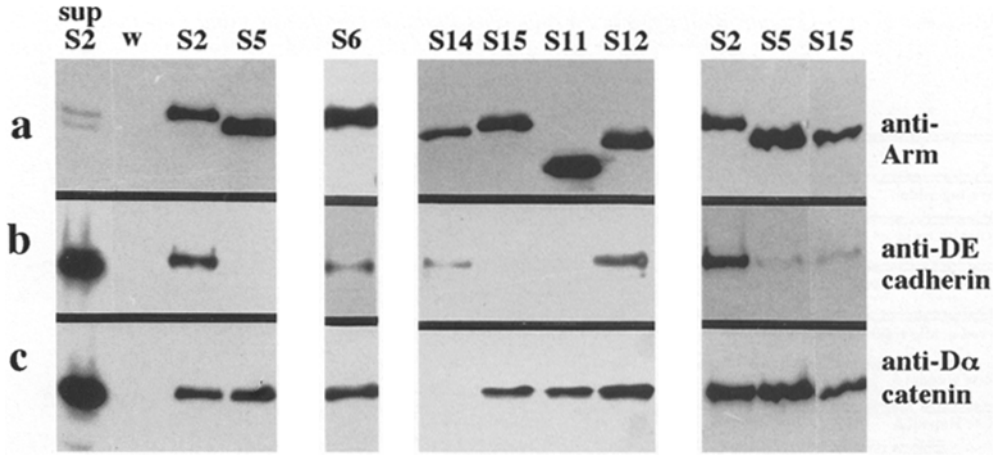


Figure 1. Different regions of Armadillo are responsible for binding to α-catenin and DE-cadherin. Anti–c-*myc* immunoprecipitates of embryonic extracts were run on SDS-PAGE and successively immunoblotted with anti-Armadillo (*a*), anti–DE-cadherin (*b*), and anti–α-catenin antibody (*c*). The first lane is the remaining supernatant after immunoprecipitation. *w* is an immunoprecipitate of control flies without myc-tagged Armadillo. Approximately the same number of embryos were used in each sample—S5 and S11 proteins are more abundant than are the other mutant proteins (*a*). S2 and S12 strongly bind to DE-cadherin, while S6 and S14 bind slightly more weakly (*b*). S5 and S15 show substantially reduced DE-cadherin binding (*b*); in some experiments they exhibited no binding and in others they retained some DE-cadherin binding (*b*; *last two lanes*). S11 never bound DE-cadherin. The proteins with the weakest binding to DE-cadherin (S5, S15, and S11) are mutants with deletions affecting the central most repeats. Only S14 (with a 39–amino acid deletion in the NH_2 terminus) failed to bind α-catenin. All other mutant proteins, including the three mutant proteins with reduced binding to DE-cadherin, coimmunoprecipitate α-catenin (*c*). Mutant proteins differ in their abundance in cell extracts, but all bind roughly the same amount of α-catenin, suggesting that for some mutants a smaller fraction is associated with α-catenin.

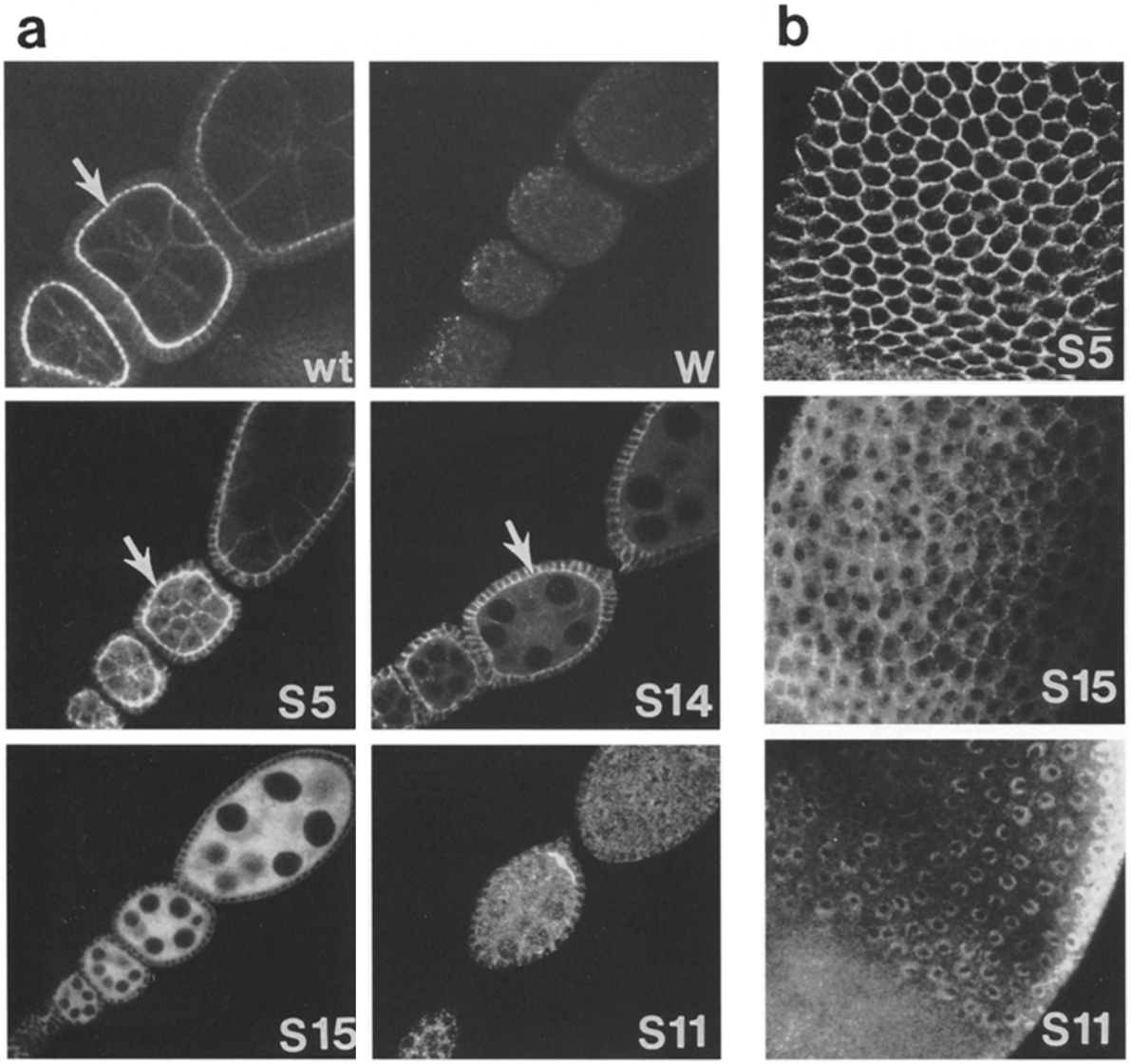


Figure 2. Certain mutant Armadillo proteins do not assemble into adherens junctions. (*a*) Localization of wild-type and mutant Armadillo proteins in ovaries in early stages of oogenesis. Wild-type Armadillo localizes to the adherens junctions of follicle cells, as visualized by anti-Armadillo antibody (*wt*, *arrow*). The other ovaries were immunostained with anti–c-*myc* antibody to visualize tagged mutant protein but not wild-type Armadillo. *W* is the negative control, containing only wild-type Armadillo, stained with anti–c-*myc*; it has only nonspecific background. S14, the α-catenin–binding mutant, localizes to adherens junctions; S14 may also show slightly reduced enrichment in the adherens junction, though it remains tightly localized to the lateral plasma membrane. S5, which has repeat 5 deleted, is found in adherens junctions but exhibits slightly higher levels of cytoplasmic staining than wild-type. S15, with a deletion of repeat 8, localizes mainly in the cytoplasm, while nuclei are devoid of S15. S11, which has four repeats deleted, exhibits faint cytoplasmic staining with no localization to a specific cellular compartment in early stages of oogenesis. (*b*) Surface views of follicle cells enveloping the oocyte at stage 10. S5 localizes to adherens junctions, similar to wild-type Armadillo. Although some S15 is associated with adherens junctions, most is in the cytoplasm, while it is excluded from nuclei. No S11 is associated with adherens junctions at any stage of development. At this stage S11 accumulates in a perinuclear pattern.

proteins at embryonic stage 9, when the effect of Wingless signal on intracellular Armadillo accumulation is most prominent (Fig. 4). We looked in a background also containing wild-type Armadillo, to maintain normal Wingless signaling, and used anti–c-*myc* Ab to distinguish mutant from wild-type Armadillo. S2 (myc-tagged but otherwise wild-type), S6 (with a mutation in repeat 1), and S12 (with repeats 10 and 11 fused) all accumulate in an essentially wild-type pattern (data not shown).

In contrast, mutant proteins with deletions in the central repeats (S5, S15, and S11) or lacking the NH_2-terminal α-catenin–binding site (S14) all localize prominently to cell nuclei. In cells responding to Wingless, wild-type Armadillo is found at relatively equal levels in the nucleus and cytoplasm (Fig. 3). In contrast S14, S5, S15, and S11 accumulate at higher levels in nuclei than in the cytoplasm (Fig. 4). These four proteins differ, however, in their response to Wingless signal. S14 (with an NH_2-terminal deletion), S5, and S15 (lacking single central repeats) respond to Wingless signal, accumulating in nuclei only of cells re-

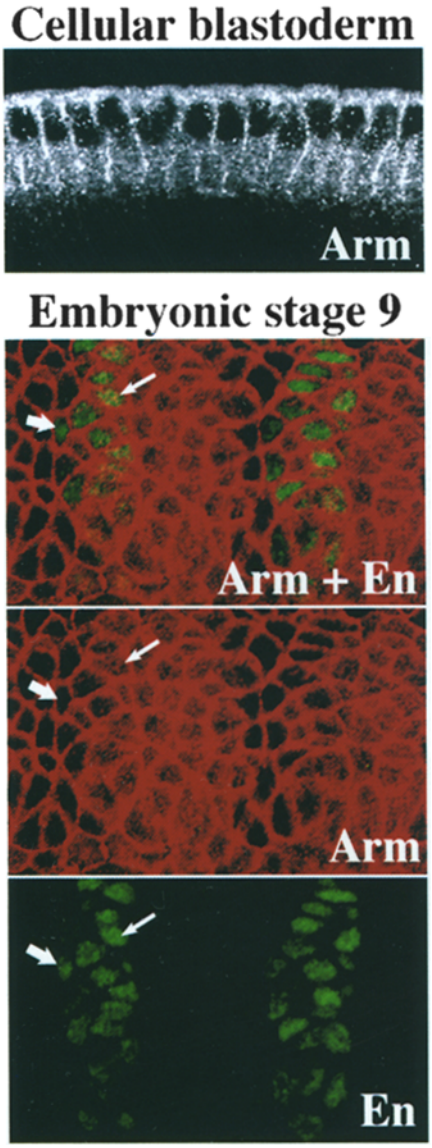


Figure 3. In response to Wingless signal, wild-type Armadillo accumulates in both the nucleus and the cytoplasm. (*Top panel*) Cross-section through a blastoderm-stage embryo stained with anti-Armadillo antibody. At this stage, preceding the onset of Wingless signaling, Armadillo accumulates in the cytoplasm of all cells and is enriched at cell–cell boundaries, but is excluded from nuclei. (*Bottom three panels*) Confocal section through a stage nine embryo, when Wingless signaling is active; the embryos were double labeled with anti-Armadillo antibody (*red*) and anti-Engrailed antibody (*green*). Engrailed is a transcription factor that accumulates in the nuclei of cells of the posterior compartment. A few of the most posterior Engrailed-expressing cells lie outside the Armadillo stripe (*fat arrow*, green color in double label). Most Engrailed-expressing cells receive Wingless signal, and thus accumulate intracellular Armadillo. In these cells, Armadillo and Engrailed colocalize (*thin arrow*, yellow color in double label), demonstrating that Wingless induces Armadillo accumulation in both the cytoplasm and in nuclei.

ceiving Wingless signal. We confirmed this by examining S5 localization in *wingless* mutants. In *wingless* mutants wild-type Armadillo fails to accumulate in the cytoplasm or nuclei of any cells (Riggleman et al., 1990; Peifer et al., 1994*b*); likewise, in *wingless* mutants S5 does not accumulate inside nuclei (Fig. 4 *c*). In contrast, S11, lacking multiple central repeats, is not strongly affected by Wingless signal, accumulating at high levels in the nuclei of all cells (Figs. 4, *a* and *b*; 5, *b* and *c*).

S5, S15, and S14 require Wingless signal for nuclear localization, while S11 does not. Wingless signal is active in the ectoderm from stages 9-12 (Bejsovec and Martinez-Arias, 1991). S11 nuclear localization is Wingless independent, as it is seen at all stages from preblastoderm through the latest stage we could examine (Figs. 4, *a* and *b*; 5, *b* and *c*). In contrast, S15 is diffusely cytoplasmic at the blastoderm stage, becomes nuclear localized at stages when Wingless signaling is active, and becomes diffusely cytoplasmic again at a later stage (Figs. 4, *a* and *b*; 5, *b* and *c*). Likewise, S5 and S14 are predominantly membrane-associated at early and later stages, becoming nuclear only at stages of active Wingless signaling (Figs. 4, *a* and *b*; 5, *a* and *b*; data not shown). Nonnuclear localization of these mutant proteins parallels the results seen in ovaries. S14 is associated with the membrane at most embryonic stages, reflecting its ability to bind cadherin and localize to adherens junctions in ovaries. For proteins mutated in the central repeats, the degree of mislocalization parallels defects in cadherin binding. S5 protein, missing repeat 5 and with reduced cadherin binding, is found at near normal levels in adherens junctions and also at abnormally high levels in nuclei (Figs. 4, *a* and *b*; 5 *a*). S15, lacking repeat 8 and reduced for cadherin binding, is primarily localized to the cytoplasm and nuclei, with a small amount in adherens junctions (Fig. 4, *a* and *b*). In contrast, S11 protein, missing multiple repeats and unable to bind cadherin, is localized exclusively to the cytoplasm and nuclei (Fig. 4, *a* and *b*).

Some Mutations Nearly or Fully Complement a Null Mutation

We subjected each mutant to a series of genetic tests designed to test function in Wingless signaling and in adherens junctions (details of the crosses involved are presented in Materials and Methods). We first tested the ability to complement a zygotic null *arm* mutation. S2 (wild-type Armadillo with a myc-tag) and S4 (with an NH_2-terminal point mutation) fully rescue a null *arm* mutant (arm^{YD35}) to adult viability and fertility. S8 (with the COOH terminus truncated) retains nearly wild-type function (one of three lines tested rescued null mutant flies to adulthood; two were lethal as pharate adults). Most of the rescued adults were normal, although imaginal disc defects similar to those seen with slight reduction in *arm* function (Peifer et al., 1991) were observed at a low frequency. These three mutants that rescue flies to adult viability were also fully functional in all other genetic tests we used, and thus we do not mention them below.

The other mutations are embryonic or early larval lethal in a zygotic null *arm* background (Fig. 6; Table II). S14 completely complements the segmentation defect of arm^{YD35}, restoring the normal alternating bands of denticles and na-

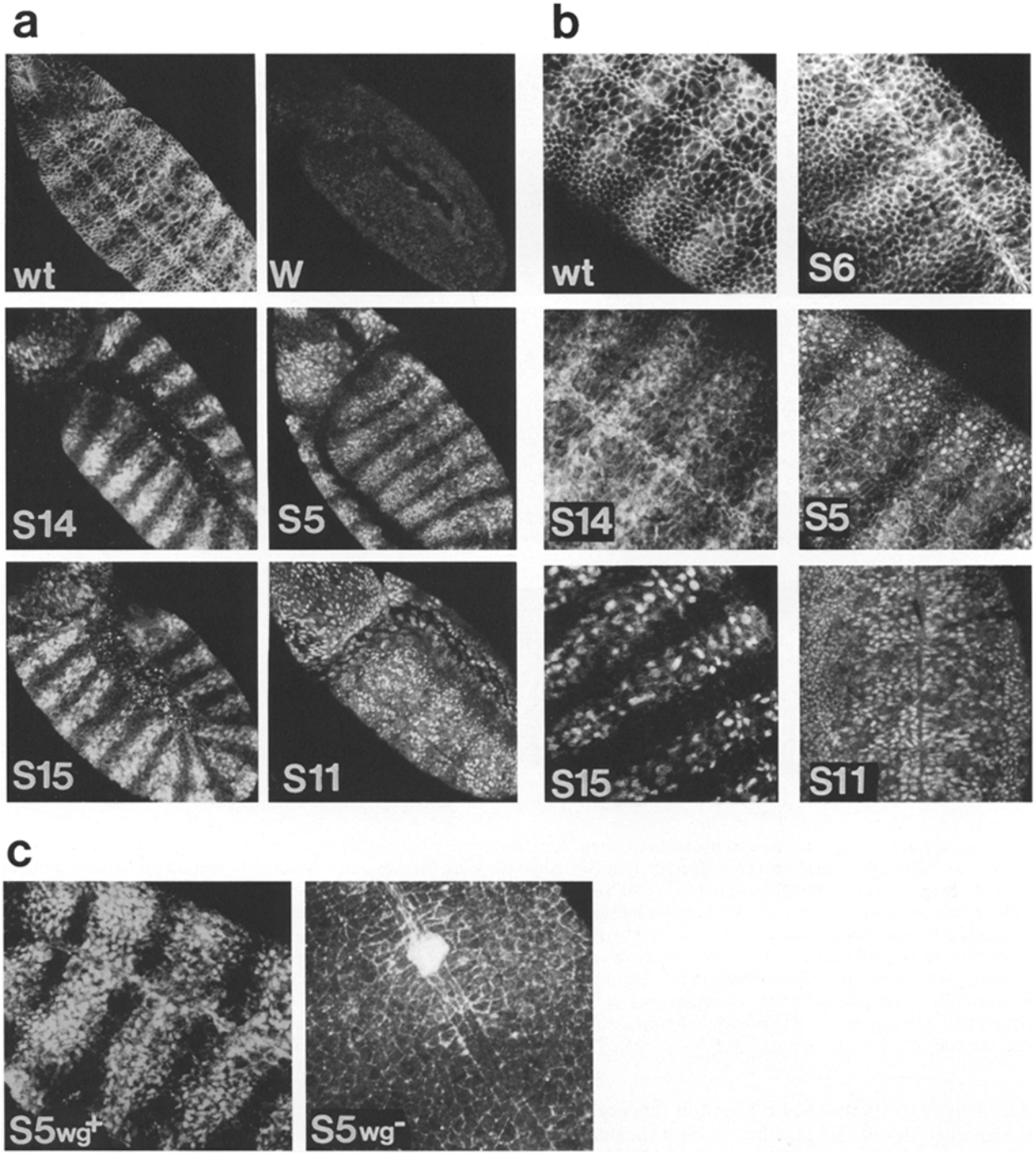


Figure 4. Armadillo mutants that are nonfunctional in adherens junctions localize to nuclei in cells that receive Wingless signal. Embryos carrying a wild-type *armadillo* gene plus a transgene encoding the Armadillo mutant protein indicated, were prepared for immunofluorescence and confocal microscopy. Wild-type (*wt*) Armadillo was detected with anti-Armadillo antibody, while mutant proteins were detected with anti-myc antibody. The anti-myc staining is specific for the tagged proteins, as it is not detected in control embryos lacking myc-tagged Armadillo (*W*). (*a*) Localization of wild-type and mutant Armadillo proteins in wild-type stage 9 embryos. (*b*) Close-ups of several segments of embryos at the same stage. Wild-type Armadillo localizes to adherens junctions of all cells, but also accumulates inside cells receiving Wingless signal; it accumulates at relatively equal levels in the cytoplasm and nuclei. S6 localization resembles wild-type. S14 and S5 mutant proteins accumulate at the membrane and also accumulate prominently in nuclei receiving Wingless signal—the ratio of mutant protein in nuclei to that in the cytoplasm is much higher than is seen for wild-type Armadillo. S15 protein accumulates mostly in nuclei; little membrane-bound protein is observed. In S5, S14, and S15, nuclear localization is the most prominent in stage 9, when Armadillo stripes are the most prominent in wild-type embryos. S11 protein accumulates both in the cytoplasm and the nuclei of most cells in the embryo, independent of whether cells receive Wingless signal. (*c*) The stripes of S5 mutant protein in nuclei disappear in a *wingless* mutant embryo, but membrane-localized S5 protein remains. This is similar to the effect of *wingless* mutations on wild-type Armadillo.

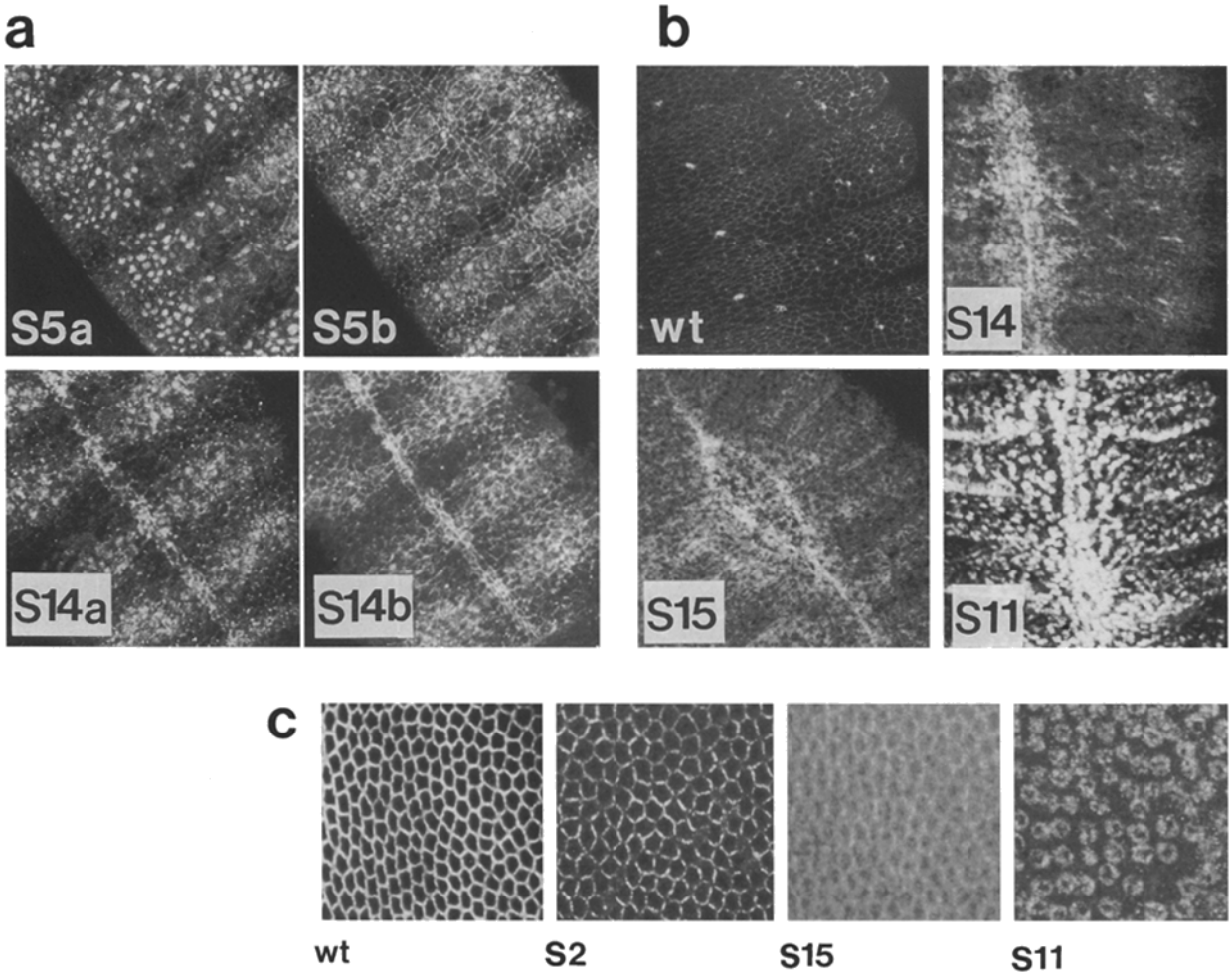
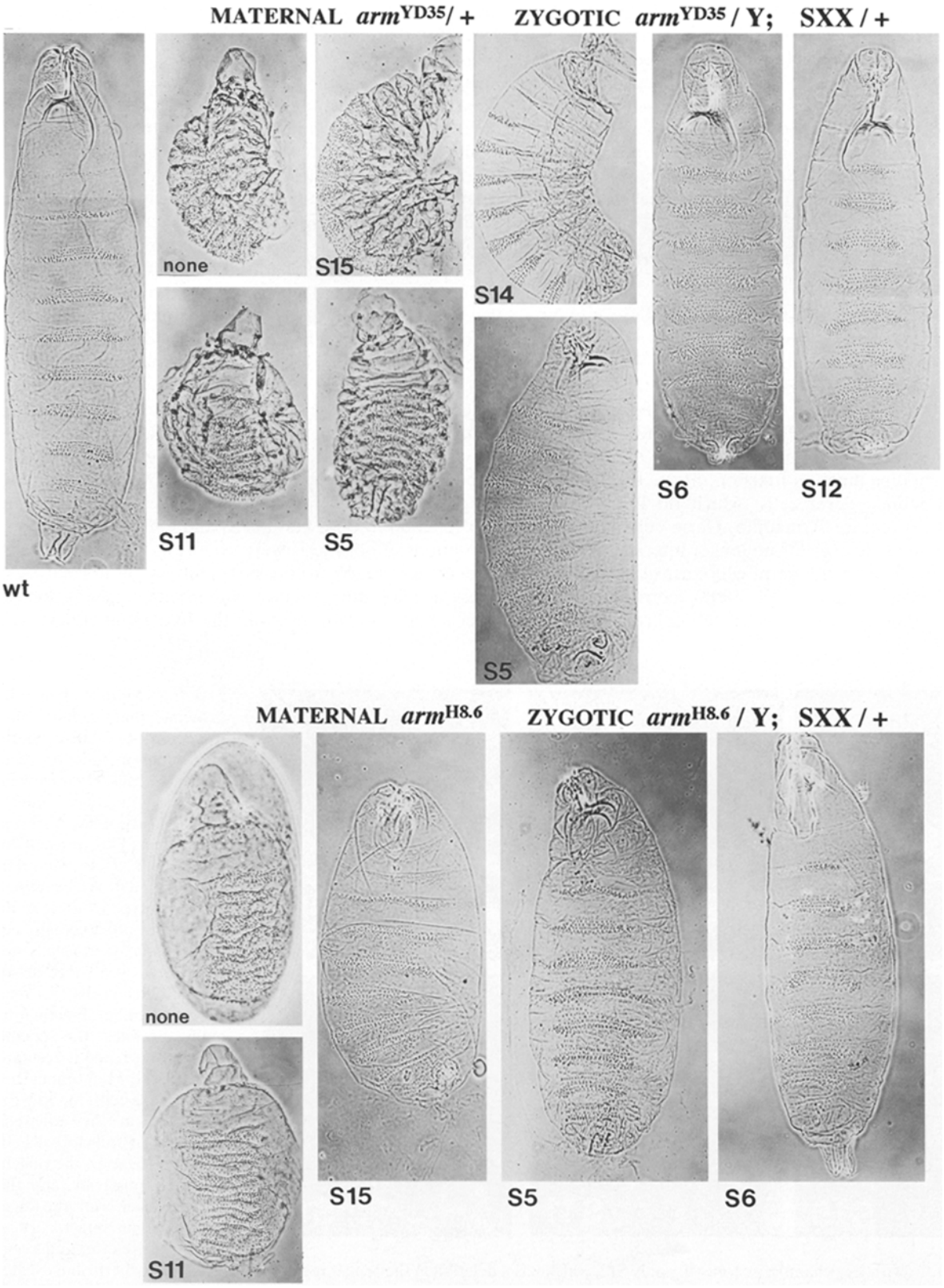


Figure 5. Certain mutant proteins localize to nuclei only when Wingless signal is active. Embryos were prepared for immunofluorescence with anti-c-*myc* antibody to detect myc-tagged mutant Armadillo proteins. (*a*) At stage 9, S5 and S14 mutant proteins accumulate in the nuclei of cells that receive Wingless signal (S5a and S14a) and in the membrane of all cells (S5b and S14b). Two different focal planes of the same embryos are shown. (*b*) S11 remains localized to nuclei late in development while other mutants do not. At stage 14, when Wingless is no longer required in the epidermis, wild-type Armadillo accumulates preferentially at the cell membrane (*wt*). At this stage, S14 and S15 no longer localize to nuclei. S11, however, remains in the nuclei of most cells. (*c*) S11 is localized to nuclei before the blastoderm stage, while other mutant proteins are not. During the cellular blastoderm stage, preceding the onset of Wingless signaling, wild-type Armadillo is associated with the membrane (*wt*), as is S2. A fraction of S15 may associate with the membrane, although a large pool of cytoplasmic S15 is present—S15 is excluded from nuclei. S11 protein accumulates preferentially in nuclei, but is apparently excluded from the nucleoli.

ked cuticle. This suggests that S14 retains full function in Wingless signaling, despite its inability to bind α-catenin and its defects in adherens junction function. However, S14 remains embryonic lethal, with severe defects in head involution and occasionally a slight dorsal closure defect (Fig. 6). We believe this is due to subtle defects in junction function, as this phenotype closely resembles that of moderate mutations in *shotgun* (Uemura et al., 1996; Tepass et al., 1996), the gene encoding DE-cadherin. This does not reveal the full defect of S14 in junction function, due to the wild-type maternal contribution of Armadillo. As we show below, S14 is nearly null for adherens junction function.

S5, S15, and S11, with mutations in central Arm repeats, are all embryonic lethal with segment polarity defects. Mutations in different repeats have distinct phenotypes. S5 (missing repeat 5) has a moderate to strong segment polarity phenotype, S15 (missing repeat 8) has a strong segment polarity phenotype, and S11 (missing repeats 3-6)

Figure 6. Mutant Armadillo proteins differ in their abilities in Wingless signaling. Details of crosses used to generate the genotypes displayed are found in Materials and Methods. (*Top panel*) Cuticle preparations of embryos with maternal wild-type Armadillo, and zygotically only the mutant Armadillo protein indicated (arm^{YD35} is a protein null). In wild-type embryos (*wt*) ventral denticle bands alternate with naked cuticle in each segment. In an *arm* zygotic null mutant (*none*), all cells secrete denticles, the embryo is shortened and has severe head and dorsal closure defects. The three mutants with deletions in central Arm repeats (S5, S11, and S15) all have severe segment polarity defects similar to (S11) or slightly weaker than (S5 and S15) the null phenotype. The S5 experiment yielded two different phe-



notypic classes; the weaker phenotype may represent embryos with one copy of wild-type *arm* and one copy of S5; S5 may have a partially penetrant dominant negative effect. S6 and S12 have very weak segment polarity defects. S14 does not have segment polarity defects, but head structures are missing. S2, S4, and S8 fully rescue the zygotic null and thus are not shown. (*Bottom panel*) Cuticle preparations of embryos maternally homozygous for $arm^{H8.6}$, and with both $arm^{H8.6}$ and SX zygotically. This assay measures the ability of mutant Armadillo proteins to replace $arm^{H8.6}$ in Wingless signaling. Embryos with maternal and zygotic $arm^{H8.6}$ have severe segment polarity defects. S11 is null in this assay, while S15 and S5 substantially rescue the phenotype and S5 is more active. S6 is nearly wild-type for Wingless signaling, with slight defects in patterning. S2, S4, S8, S12, and S14 fully rescue the Wingless signaling defect.

has a very strong segment polarity defect, indistinguishable from that of a null *arm* allele (Fig. 6). In contrast, mutations in other repeats have less severe consequences. S6 (a point mutation in repeat 1) and S12 (repeats 10-11 fused) are zygotically lethal as first instar larvae (a fraction die as embryos), with a very weak segment polarity phenotype (Fig. 6), suggesting that S6 and S12 are nearly fully functional in junctions, and retain some function in Wingless signaling, a conclusion substantiated by other tests below.

Mutations that Block α-Catenin and Cadherin-binding Block Junction Function

To examine the adherens junction function of our mutant proteins, we tested each in two assays that allow measurement of junction function separately from Armadillo's role in Wingless signaling (Figs. 7 and 8; Table I; see Materials and Methods for details of crosses involved). Mutations affecting α-catenin or DE-cadherin binding reduce or eliminate adherens junction function, while other mutations leave adherens junction function intact. The first assay involved female germ cells, which do not require Wingless but do require Armadillo. Germ cells homozygous for the null allele arm^{YD35} no longer interact with adjacent follicle cells, and the germ cell cortical actin cytoskeleton collapses (Peifer et al., 1993), consistent with disruption of cadherin-catenin based cell interactions. We introduced mutant Armadillo proteins into this background to determine if the oogenesis defects were rescued (Fig. 7).

S14, defective in α-catenin binding, has severe junctional defects. The mutations in S5, S11, and S15, affecting the central repeats, all disrupt junction function, and do so to an extent consistent with the strength of their defects in DE-cadherin binding (Figs. 7 and 8; Table I). S11, with a deletion of four repeats, and S14, missing the α-catenin–binding site, are null for junction function in this assay. Ovaries in which the germline is homozygous for S11 or S14 have defects indistinguishable from those seen when germ cells are homozygous for the null allele, arm^{YD35}; these females lay greatly reduced numbers of defective eggs, none of which begin embryonic development (Fig. 7). S15 retains a very small but detectable amount of junction function in the oogenesis assay; egg chambers in which S15 is the only Armadillo protein share disruptions similar to those of the null allele (Fig. 7), but a small number of the eggs laid by these females began embryonic development, and later had severe disruptions in epithelial integrity (Fig. 8). S5 has weaker effects on adherens junction function in both germ cells and embryos. Occasional disruptions of oogenesis were seen (Fig. 7), but S5 mutant females lay nearly wild-type numbers of normal eggs. These develop into embryos with severe segment polarity defects and also with holes in the remaining cuticle, sug-

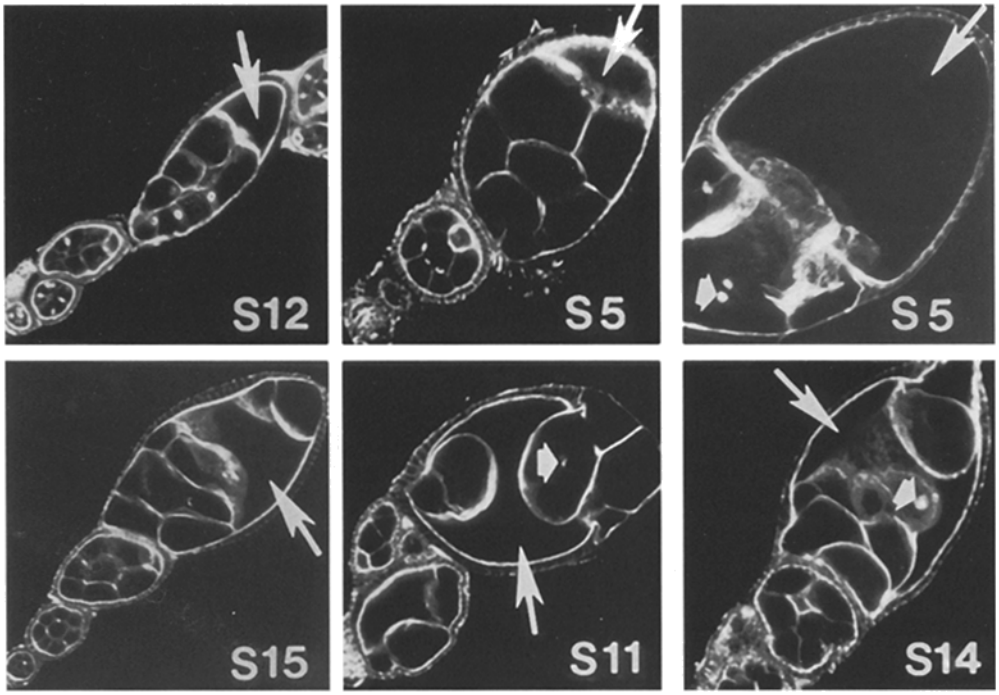


Figure 7. Mutant Armadillo proteins that cannot bind α-catenin or DE-cadherin lack adherens junction function in ovaries. Ovaries were generated in which mutant Armadillo proteins were the only Armadillo present in germ cells (see Materials and Methods). We examined the phenotype of these germ cells using phalloidin to detect F-actin. Germ cells are enveloped by a sheet of somatic follicle cells, forming the egg chamber. F-actin normally outlines the oocyte (*thin arrows*), and it accumulates in the membranes between nurse cells, the ring canals, and in the adherens junctions of follicle cells. In wild-type ovaries, the oocyte is always posterior, and the nurse cells anterior; the nurse cells have an intact cortical actin cytoskeleton and regular shapes (not shown). Egg chambers expressing only S12, which has a deletion in the repeat region, but binds DE-cadherin and α-catenin, have wild-type morphology. In contrast, egg chambers expressing only mutant Armadillo proteins that do not bind either DE-cadherin (S5, S15, and S11) or α-catenin (S14) often have an altered morphology, exhibiting disrupted cell adhesion. More than 50% of the egg chambers expressing only S14 or S11 have obvious defects. S5 and S15 show weak DE-cadherin binding. More than 50% of the egg chambers expressing S5 and ~50% of the egg chambers expressing S15 have a wild-type morphology. The remaining egg chambers exhibit alterations in nurse cell shape, fusion of nurse cells, aggregation of actin (*fat arrows*), or rearrangement of cells inside the egg chamber such that the oocyte can be in the middle (*thin arrows*) or at the anterior end of the egg chamber. The disruptions are similar to those seen in egg chambers in which germ cells have no functional Armadillo (Peifer et al., 1993). All egg chambers expressing only S11 have a disrupted morphology.

gesting defects in epithelial integrity (Fig. 8). Thus, a deletion of repeat 5 is much less deleterious for junction function than a deletion of repeat 8. S6 (with a point mutation in repeat 1; data not shown) and S12 (with repeats 10 and 11 fused), which show nearly wild-type α-catenin and cadherin binding, retain full adherens junction function in this assay (Fig. 7; Table I).

We also examined Armadillo function in epithelia in the embryonic ectoderm. While we cannot completely remove maternal Armadillo (since oogenesis is disrupted), we can substantially deplete maternal Armadillo function using an intermediate allele, arm^{XP33}. Embryos containing only arm^{XP33} protein make a cellular blastoderm, but this epithelium disintegrates as the cells separate and become mesenchymal (Cox et al., 1996). The few cells retaining epithelial character secrete small pieces of cuticle. This allows us to test junction function in an epithelium before Armadillo's role in Wingless signaling comes into play. We introduced each mutant into this background.

The results of the arm^{XP33} assay were nearly identical to those of the oogenesis assay. Mutations with deletions of central repeats (S5, S11, and S15) or defects in α-catenin binding (S14) disrupt junction function to varying degrees (Figs. 7 and 8; Table I). In the blastoderm epithelium, S11, lacking multiple central repeats, and S15, missing repeat 8, were null for junction function. S14, defective in α-catenin binding, retained very slight function in junctions, evidenced by a slight increase in the number of cuticle fragments made (Fig. 8). S5, with repeat 5 deleted, has only small holes in the cuticle, and thus retains substantial adherens junction function (Fig. 8).

The Central Most Repeats Are Crucial for Wingless Effector Function

We also measured the function of each mutant in Wingless signaling in isolation from its junction function (see Materials and Methods for details of crosses involved). To do so, we used $arm^{H8.6}$ which lacks the COOH terminus. $arm^{H8.6}$ is fully functional in junctions but severely impaired in transduction of Wingless signal (Peifer et al., 1991, 1993). We generated females with germlines homozygous for $arm^{H8.6}$ and crossed them to males carrying each mutant protein. Half of the progeny are maternally and zygotically $arm^{H8.6}$ mutant, and also carry the introduced mutant *arm* gene. We tested the ability of mutant proteins to complement the $arm^{H8.6}$ defect in Wingless signaling, while relying on $arm^{H8.6}$ to provide junction function (Fig. 6; Table II).

S14 fully rescues $arm^{H8.6}$; larvae hatch with normal segment polarity, confirming that S14, despite deficits in α-catenin binding and adherens junction function, provides full Wingless signaling activity. The other mutant proteins have varying degrees of impairment of Wingless signal transduction. In this assay, S6 (mutant in repeat 1) and S12 (with repeats 10-11 fused) are wild-type or nearly wild-type for Wingless signaling (Fig. 6). Mutants with deletions of central Arm repeats are more severely affected. S5, lacking repeat 5, is embryonic lethal, with a mild segment polarity defect. S15, lacking repeat 8, is more strongly defective in Wingless signaling. S11, missing repeats 3-6, does not rescue $arm^{H8.6}$ at all, suggesting that S11 is nearly completely or completely defective in its ability to transduce Wingless signal (Fig. 6).

Since S5, S6, and S12 retain some adherens junction function, we can also create embryos in which both the maternal and zygotic contribution is mutant protein (Fig. 8; Table II).This is our most stringent test for function in Wingless signaling. Each mutant had a more severe segment polarity phenotype than was seen in assaying their ability to rescue $arm^{H8.6}$. S5, lacking repeat 5, had a null segment polarity phenotype and also had holes in the remaining cuticle (Fig. 8), suggesting that S5 is somewhat defective in junction function and strongly defective in Wingless signaling. Embryos in which S6 and S12 were the only Armadillo protein had moderate segment polarity defects (Fig. 8), suggesting moderate defects in Wingless signal transduction in these mutants.

Armadillo's Roles in Adherens Junctions and Wingless Signaling Are Separable

One critical question is whether Armadillo's roles in adherens junctions and in Wingless signaling are identical or separable. Most data supports separable roles (for review see Peifer, 1995). Among the evidence is our demonstration that mutant proteins like that encoded by $arm^{H8.6}$, lacking Armadillo's COOH-terminal domain, retain function in adherens junctions but lack function in Wingless signal transduction. Here we identified a mutation with complementary properties. S14 lacks Armadillo function in adherens junctions but retains its function in Wingless signaling (Fig. 6). If Armadillo's roles in adherens junctions and Wingless signaling are separable, S14 and $arm^{H8.6}$ should complement each other, restoring wild-type development. To test this we crossed S14 into the $arm^{H8.6}$ background. While both $arm^{H8.6}$ and S14 are embryonic lethal at 25°, their combination in a single animal rescued flies to adult viability, normal pattern, and fertility. This interallelic complementation supports the suggestion that the two roles of Armadillo are separable; each can be carried out by a separate protein when both proteins are present in the same cell.

Possible Dominant Negative Effects

One concern we had when initiating these experiments was that certain mutants might have dominant negative effects. Since we expressed mutants with the endogenous *armadillo* promoter, they were expressed ubiquitously and at high levels; if a mutant protein had a strong dominant negative effect we would have failed to obtain transformants of the construct encoding it, or those transformants would not express mutant protein. However, no substantial dominant negative effects were seen for any of the mutant proteins described here, although all are expressed at or above the level of endogenous wild-type Armadillo. Wild-type Armadillo normally accumulates to very high levels and thus its functional concentration is not likely to be limiting—weak dominant negative activity might not be detected in the presence of normal levels of wild-type Armadillo.

In situations where levels of Armadillo or Wingless were reduced, however, we observed subtle effects with certain mutants that may be due to dominant negative ac-

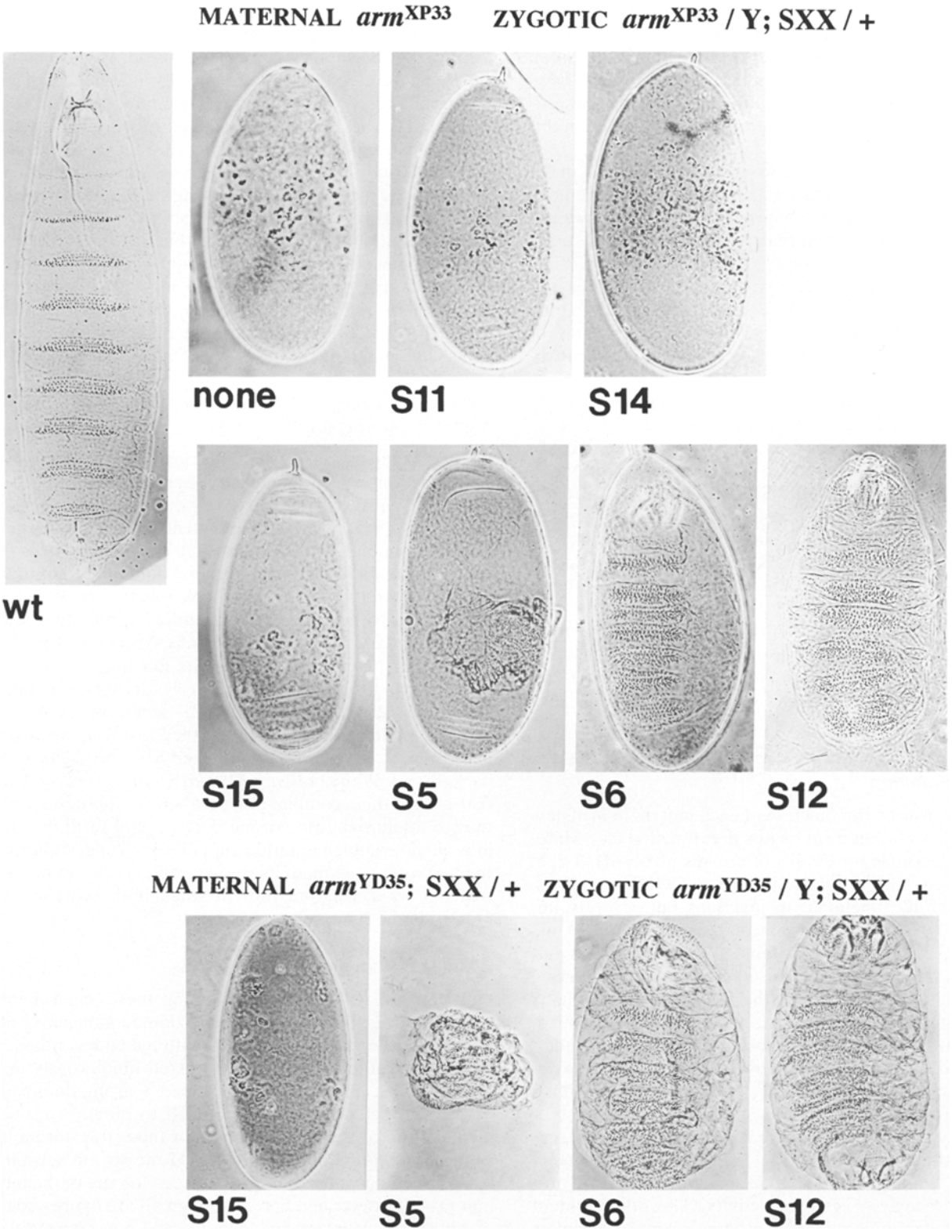
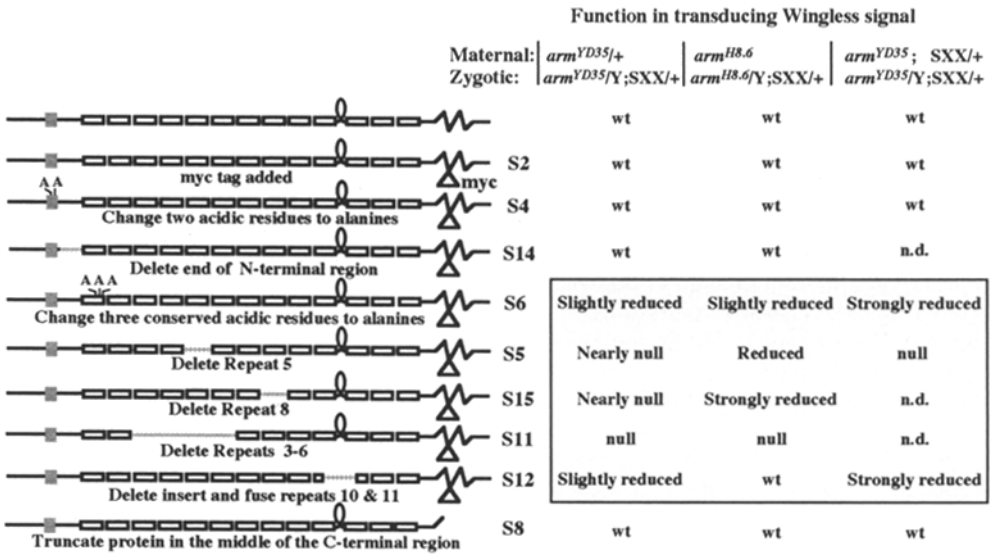


Figure 8. Mutant Armadillo proteins that do not bind α-catenin or DE-cadherin lack junction function. Details of crosses used to generate the genotypes displayed are found in Materials and Methods section. (*Top panel*) Cuticle preparations of embryos that received arm^{XP33} maternally and zygotically; the mutant protein indicated was also introduced zygotically. Embryos maternally and zygotically arm^{XP33} mutant (none) secrete only small bits of cuticle. S11 and S15 do not rescue this disruption of junction function, while S14 rescues it very slightly (more scraps of cuticle). S5 rescues junction function substantially but not completely (note cuticle holes), however, the embryos have severe segment polarity defects. S6 and S12 rescue junction function, but have moderate defects in Wingless signaling. (*Bottom panel*) Embryos in which maternal and zygotic Armadillo consist entirely of the mutant protein indicated. S15 maternally and zygotically mutant embryos secrete small bits of cuticle. Embryos containing only S5 have severe segment polarity defects and holes in the remaining cuticle. S6 and S12 have moderate segment polarity defects, but are wild-type for adherens junction function.

Table II. Effects of Mutations on Ability to Transduce Wingless Signal



n.d. - this maternal genotype does not lay eggs or the embryos do not make much cuticle, so it is impossible to assess the segment polarity phenotype.

tivity. We introduced our mutant Armadillo proteins into embryos in which the only maternally contributed Armadillo was $arm^{H8.6}$ mutant protein, greatly reducing the level of Armadillo function in Wingless signaling. Normally, 50% of these embryos die with strong segment polarity defects (those animals that are maternally and zygotically mutant); the others are zygotically rescued by the paternal wild-type gene and hatch as larvae. When we introduced into this genetic background mutant constructs with deletions of central Arm repeats (S5, S11, or S15), however, some embryos which should have been zygotically rescued instead died with a segment polarity defect (60–72% lethality; Table III). We also observed adult patterning defects in animals heterozygous for either a null *wingless* mutation or a null *armadillo* allele that also expressed S5 or S11 (data not shown).

Phosphorylation and Accumulation of Mutant Armadillo Proteins

Anti-Armadillo antibodies recognize different Ser/Thr phosphorylation isoforms of Armadillo (kD = 105–115; Peifer et al., 1994*a*). The most rapidly migrating isoform is least phosphorylated, while successively slower migrating forms represent more phosphorylated variants (Fig. 9). More highly phosphorylated Armadillo isoforms are membrane-associated while less phosphorylated isoforms are

Table III. Possible Dominant Negative Effects

SXX construct added	Ratio of embryos Mutant/wild-type	Number of embryos examined
None	0.98 (Expected = 1.0)	475
S5 line 1	2.62	448
S5 line H3	2.68	81
S15 line F	2.10	319
S15 line E	2.43	168
S11 line E	1.75	283
S11 line A	1.53	481

Females with germlines homozygous for $arm^{H8.6}$ were crossed to SXX males. Embryos were zygotically $arm^{H8.6}/Y$; *SXX/+* or $arm^{H8.6}/+$; *SXX/+*.

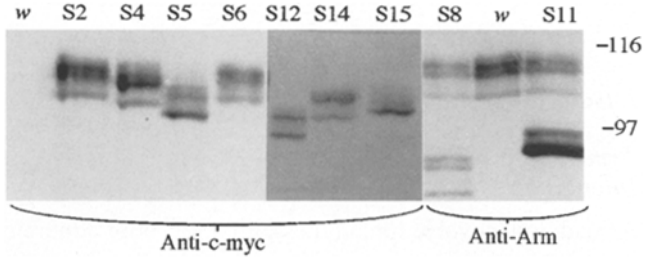


Figure 9. Certain *armadillo* mutations alter protein phosphorylation and stability. Protein extracts from wild-type (*w*) or mutant embryos were analyzed by SDS-PAGE and immunoblotting with either anti–c-*myc* or anti-Armadillo antibody. This allows visualization of a subset of Armadillo's Ser/Thr phosphorylation isoforms; the fastest migrating band is a hypophosphorylated form of Armadillo, while more slowly migrating bands are more highly phosphorylated. In all cases, several independent transformant lines were examined, and the lines shown are representative. Anti-Armadillo antibody recognizes both wild-type and myc-tagged mutant proteins, while anti–c-*myc* antibody specifically recognizes only mutant protein. S8, which lacks the c-*myc* tag, is distinguishable from wild-type Armadillo by its size. S2 migrates more slowly than wild-type Armadillo due to the c-*myc* tag. S11 is shown with anti-Armadillo antibody so that its level of accumulation can be compared to the wild-type. Mutant proteins show different patterns of phosphorylation. Phosphorylation of S2, S4, S6, S8, and S14, as assayed by the levels of different isoforms seen compared to wild-type, is normal. S12 phosphorylation is slightly reduced, while S15 and S5 are even less phosphorylated. S11 phosphorylation is very strongly reduced. All mutant proteins are stable and expressed at least to the same level as wild-type Armadillo. S11 accumulates to higher levels in embryos than the wild-type protein; S5 may also accumulate to slightly higher levels.

cytoplasmic (Peifer et al., 1994*a*). Phosphorylation may regulate Armadillo's role in Wingless signaling and could also modulate its role in cell adhesion.

We examined each mutant for the subset of phosphorylation isoforms detectable by SDS-PAGE (Fig. 9). Mutations in either the NH_2- or COOH-termini do not alter phosphorylation as detected by this assay. S2 protein migrates more slowly than untagged Armadillo (Fig. 9), due to the 13–amino acid c-*myc* tag insertion, but the tag does not alter its state of phosphorylation. Although truncation of the COOH-terminal region in S8 deletes several potential phosphoacceptors, no apparent change in phosphorylation was detected (Fig. 9). Similarly, mutations in the NH_2-terminal region of S14 or repeat 1 of S6 have no apparent effect on phosphorylation (Fig. 9).

Phosphorylation changes were detected only in mutant proteins with deletions in the Arm repeats. Mutant proteins lacking repeat 5 (S5), repeat 8 (S15), or repeats 3-6 (S11), or with repeats 10 and 11 fused (S12) were less phosphorylated than normal, as revealed by the increase in abundance of the fastest migrating isoforms (Fig. 9). Changes in phosphorylation levels varied in degree; phosphorylation of S12 was only slightly decreased while S11 phosphorylation was dramatically altered. While most mutant proteins accumulate at levels similar to wild-type, S11 accumulates at higher levels (Fig. 9; S5 also appears to accumulate at slightly higher levels; these differences were observed in several independent transformants of both S5 and S11). This may be due to a higher stability of these mutant Armadillo proteins. Three of four hypophosphorylated mutant proteins exhibit dramatically altered localization in embryos (see above).

Discussion

Armadillo Is a Mosaic of Protein–Protein Interaction Motifs

Armadillo is involved in, and essential for, both adherens junctions and Wingless signaling. We suspected that particular regions of Armadillo might be primarily, if not exclusively, involved in one or the other function. Our data show that distinct regions of Armadillo mediate association with α-catenin and DE-cadherin; both interactions are critical for adherens junction function (Fig. 10). Several sites are required for Wingless effector function, including the central repeat region; this region may contain overlapping binding sites for cadherins and for the Wingless effector.

DE-cadherin and α-Catenin Interact with Armadillo Independently of Each Other and the Presence of Both Proteins Is Crucial for Adherens Junction Function

Armadillo, with its multiple degenerate Arm repeats, is a linear mosaic of protein–protein interaction motifs, each somewhat independent of the others. Deletion of nearly half of Armadillo does not totally eliminate function (Peifer and Wieschaus, 1990); certain deletions affect some functions and leave others untouched. Our mutational approach was based on this model. It was designed to remove binding sites for particular partners, allowing us to assign specific functions to particular Armadillo domains. This expectation was largely met, although as we discuss below, certain regions of Armadillo appear to contain overlapping binding sites for different partners.

We identified the binding sites on Armadillo of DE-cadherin and α-catenin in vivo (Fig. 10). α-Catenin binding requires the distal NH_2 terminus, consistent with data derived from our in vitro binding experiments, which define a 77–amino acid region at the junction of the NH_2 terminus and repeat 1 as necessary and sufficient for binding (Pai, L.-M., C. Kirkpatrick, and M. Peifer, unpublished data). This is also consistent with data from the vertebrate system (Sacco et al., 1995; Aberle et al., 1996). DE-cadherin binding requires the central Arm repeats. This is also consistent with our in vitro binding data (Pai, L.-M., C. Kirkpatrick, and M. Peifer, unpublished data), which suggest that repeats 3-8 form the minimal cadherin-binding site, and with similar data from the vertebrate system (Aberle et al., 1994; Hülsken et al., 1994; Oyama et al., 1994; Jou et al., 1995).

Our data show that Armadillo can interact independently with α-catenin and DE-cadherin, but that each interaction is required for adherens junction function. Cell adhesion is disrupted when α-catenin is absent from adherens junctions due to mutation of Armadillo's α-catenin–binding site. The zygotic S14 phenotype is similar to that of a moderate cadherin mutant (Fig. 6; Uemura et al., 1996; Tepass et al., 1996); more dramatic cell adhesion defects are seen if S14 is the only protein expressed mater-

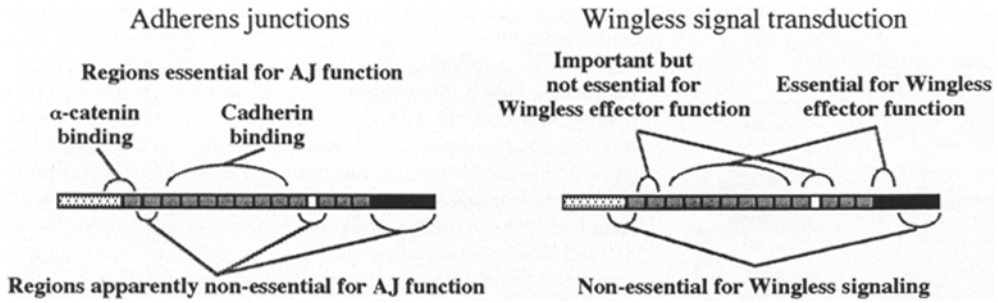


Figure 10. Summary of the regions of Armadillo protein that are required for adherens junction function and Wingless signal transduction. The binding site for α-catenin is at the junction of distal NH_2-terminal region and the repeats, while repeats 3-8 are required for DE-cadherin binding; both regions are essential for function in junctions. The repeat region is also essential for Wingless signaling, although mutation of different repeats has different consequences. The proximal COOH-terminal region is also essential for Wingless signal transduction. The conserved insert between repeats 10 and 11 and repeat 1 are also involved in the Wingless signal transduction. The distal COOH-terminal region is not essential for either function of Armadillo.

nally and zygotically. The phenotype of a mutation in fly α-catenin is likely to be similar to that of S14. Our data parallel results in mammalian cells lacking α-catenin (Hirano et al., 1992): β-Catenin binds cadherin, yet without α-catenin there is not effective cell–cell adhesion.

α-Catenin links adherens junctions to the cytoskeleton (Nagafuchi et al., 1991; Herrenknecht et al., 1991; Rimm et al., 1995). *arm* mutations blocking binding of Armadillo to either α-catenin or DE-cadherin apparently block assembly of α-catenin into adherens junctions, as they result in disorganization of the cortical actin cytoskeleton (Fig. 7). Two other features of S14 are also worth noting. In epithelial follicle cells wild-type Armadillo accumulates at low levels all along the lateral cell surface but is very strongly enriched in apical adherens junctions. S14 appears slightly less enriched in junctions (Fig. 2 *a*); perhaps if the cadherin-Armadillo complex is no longer attached to the actin cytoskeleton, its assembly into the higher order adherens junction complex is diminished. Second, when S14 protein is introduced into embryos in which the only protein is provided by arm^{XP33}, S14 slightly rescues the epithelial defects (Fig. 8). Perhaps S14, deficient in α-catenin binding, and arm^{XP33}, diminished in its ability to bind cadherin, are functionally additive, generating a small amount of adhesive activity—this would suggest a more complex model than that proposing a 1:1:1 stoichiometry of cadherin:Armadillo:α-catenin.

Armadillo also binds α-catenin independently of cadherin association or assembly into adherens junctions, as mutant Armadillo proteins with defects in DE-cadherin binding retain α-catenin binding (see Fig. 1). However, cadherin binding is, not surprisingly, essential for adherens junction function in vivo. There is a good correlation between the degree to which DE-cadherin binding is reduced and the degree to which adherens junction function is compromised (Table I).

Different Arm Repeats Play Functionally Distinct Roles

The central two-thirds of Armadillo consists of 13 degenerate Arm repeats (Riggleman et al., 1989)—individual repeats share 20–30% amino acid identity. Our mutational analyses demonstrate that different repeats serve distinct but essential functions, despite their sequence similarity; differences were observed in both biochemical and biological assays. Interaction of Armadillo with DE-cadherin is reduced by removal of either repeat 5 or repeat 8, and abolished by deletion of repeats 3-6—parallel effects of these mutations on adherens junction function prove the functional importance of this binding. In contrast, mutations in repeat 1 or repeats 10-11 have little or no effect on cadherin binding or junction function. These data are in agreement with data obtained in both tissue culture and in vitro. Multiple central repeats rather than single repeats are required for cadherin binding (Aberle et al., 1994; Hülsken et al., 1994; Oyama et al., 1994; Jou et al., 1995; Pai, L.-M., C. Kirkpatrick, and M. Peifer, unpublished data). Since mutations affecting different repeats reduce but do not eliminate DE-cadherin binding, we suspect that cadherin binding is not confined to an individual repeat but to a domain formed from several different repeats, likely folded into a higher order structure. Mutations in the central repeat region also affect Armadillo's ability to both transduce and respond to Wingless signal (Fig. 10). Deletion of different repeats results in quite different phenotypes and in different intracellular distributions, suggesting that the repeats each have distinct functions. We presume these differences in phenotype reflect the fact that the repeats form multiple and sometimes overlapping binding sites for different protein partners.

Not all regions of Armadillo are crucial for function in vivo in either junctions or signaling (Fig. 10). Insertion of a c-*myc* tag into the Gly/Pro-rich region of the COOH-terminal domain or mutation of two NH_2-terminal charged amino acids (S2 and S4, respectively) had no effect on either function. Truncation of the COOH terminus in S8 also does not affect either function. The remainder of the COOH terminus is essential for Wingless signal transduction, however; S8 is only 32 amino acids longer than $arm^{H8.6}$ (Peifer and Wieschaus, 1990), yet $arm^{H8.6}$ is embryonic lethal with defects in segment polarity, while S8 is adult viable.

Armadillo Plays Independent Roles in Adherens Junctions and Wingless Signaling

Adherens junctions and Wingless signaling both use Armadillo. In *Xenopus*, β-catenin and plakoglobin can play similar dual roles (McCrea et al., 1993; Heasman et al., 1994; Funayama et al., 1995; Karnovsky and Klymkowsky, 1995; Fagatto et al., 1996). This raised the question of whether Wingless signaling acts via modulation of junctional assembly and cell adhesion, or whether the two processes, while sharing a common protein component, are independent.

We considered three possibilities: (*1*) the Wingless signal pathway is branched; one branch affects cell adhesion through Armadillo, (*2*) the components of the Wingless signal transduction pathway are localized to junctions, via interaction with Armadillo, (*3*) adherens junctions and Wingless signaling are separable; different Armadillo molecules participate in each. Available data support separable functions. In *Drosophila*, all known functions of Wingless are mediated via Armadillo, rendering unlikely the idea that Armadillo is part of one of many branches in signal transduction, influencing only adhesion. Second, in both embryos and cultured cells Wingless stabilizes the intracellular pool of Armadillo, with little effect on the junctional pool (Peifer et al., 1994*b*; van Leeuwen et al., 1994). Thus, two separate pools of Armadillo correlate with junction function and Wingless signaling. Finally, our mutational analysis identified distinct domains responsible for one or the other of Armadillo's functions. The most dramatic demonstration of this is the ability of S14 and $arm^{H8.6}$ to functionally complement; we thus constructed a fly in which Armadillo's two functions are carried out by two different proteins. These data are consistent with results obtained in Xenopus (Funayama et al., 1995; Fagatto et al., 1996); β-catenin mutants with greatly reduced C-cadherin affinity still act in Wnt signaling.

While the functions of Armadillo in adherens junctions and in Wingless signal transduction are apparently carried out by separate protein complexes in different locations, there may be cross-regulation between these pathways,

since both share a common protein component. In both *Xenopus* (Heasman et al., 1994) and *Drosophila* (Cox et al., 1996), one can manipulate cadherin and Armadillo/β-catenin dosages such that Armadillo/β-catenin's dual roles become competitive. While in *Drosophila* embryos Armadillo levels are not normally limiting, there may be situations in which cell adhesion and Wingless signaling cross-regulate via competition for available Armadillo.

This may involve competition for overlapping binding sites on Armadillo (Fig. 10). While mutation of the α-catenin–binding site does not affect Wingless signaling, mutations in the central repeats alter both cadherin-binding and Wingless signal transduction. The strength of both effects are parallel; S5 is least affected in both cadherin-binding and Wingless signaling while S11 is most affected in both. The central repeats may contain binding sites for both cadherin and the as yet unidentified Wingless effector(s). Competition for overlapping binding sites would ensure that a molecule of Armadillo was only part of one or the other complex, preventing, for example, nonproductive recruitment of Wingless effectors to adherens junctions.

The binding site on Armadillo for the tumor suppressor protein APC also resides in the central repeats, and APC and cadherin can compete for catenin binding (Su et al., 1993; Hülsken et al., 1994; Rubinfeld et al., 1995). APC regulates cytoplasmic levels of β-catenin (Munemitsu et al., 1995) and thus may be part of the Wingless signaling pathway (Rubinfeld et al., 1996). APC may be solely a negative regulator of Armadillo/β-catenin in signaling, or may be a Wingless effector. Other evidence consistent with competition in binding of different partners is found in the differences in ratios of junctional, cytoplasmic, and nuclear accumulation of mutants altering the central repeats. Reduction in the affinity of binding of Armadillo for one of its partners, e.g., cadherin or APC, may render other partners, e.g., factors in the nucleus, better able to compete for available Armadillo, altering the relative ratios of protein in different regions of the cell.

Differences in the ability to interact with APC may cause some of the striking differences seen in the pattern of accumulation of different mutant proteins. S5, S15, S14, and S11 all preferentially accumulate in nuclei. S5, S15, and S14 require Wingless signal for increased intracellular stability and thus accumulate in nuclear stripes in embryos (Figs. 4 and 5), while S11 accumulates in nuclei of all cells, regardless of whether they receive Wingless (Figs. 4 and 5). Armadillo is normally unstable outside junctions; Armadillo degradation requires the action of Zeste-white3 kinase (Peifer et al., 1994*b*), and, at least in vertebrates, APC (Munemitsu et al., 1995; Rubinfeld et al., 1996). Wingless signal counteracts this stabilizing Armadillo inside cells. S11 evades the normal destabilization mechanism. Perhaps it no longer can interact with APC, and thus is no longer degraded—this would be consistent with the rough mapping of the APC-binding site on β-catenin to the Arm repeats (Su et al., 1993; Hülsken et al., 1994; Rubinfeld et al., 1995).

Wild-Type Armadillo Accumulates in the Nucleus and May Act There to Transduce Wingless Signal

In response to Wingless signal, endogenous Armadillo accumulates not only in the cytoplasm but also in the nucleus of wild-type embryos (Fig. 3; Peifer et al., 1994*b*). S14 preferentially accumulates in nuclei rather than the cytoplasm and retains Wingless signaling function, reinforcing the possibility that the nucleus is the site of action. Levels of wild-type Armadillo are equivalent in the nucleus and cytoplasm of cells responding to Wingless, and thus its nuclear localization is not as striking as that of Armadillo mutants lacking central Arm repeats that accumulate preferentially in nuclei. Similar results were obtained in *Xenopus* (Funayama et al., 1995; Fagatto et al., 1996). Mutant β-catenin proteins that are active in Wnt signaling accumulate in the nucleus; some show little, if any, accumulation in junctions or the cytoplasm. These data raise the possibility that Armadillo's role in Wingless signaling is in the nucleus. We imagine that Armadillo might form a complex with one or more nuclear proteins, which might then influence gene expression.

Our data suggest that if Armadillo acts in the nucleus in Wingless signal transduction, it may interact with two distinct partners. As Armadillo lacks a clear nuclear localization signal, we imagine it travels into the nucleus by interaction with a nuclear-localized protein. However, this interaction is not sufficient for signaling activity, as mutants like S15 or S11 retain the ability to preferentially accumulate in nuclei yet lack signaling activity. Perhaps there is a second partner required for altering gene expression but not for nuclear accumulation.

There is one significant difference between results in *Drosophila* and *Xenopus*. In *Xenopus*, the repeat region alone possesses Wingless effector activity (Funayama et al., 1995), while in *Drosophila* both the repeat region and the proximal COOH-terminal region are required. The *Xenopus* experiments involve substantial overexpression of β-catenin. The COOH terminus may enhance activity in Wingless signal transduction; substantial overexpression may render this domain nonessential.

Phosphorylation, Wingless Signaling, and Intracellular Localization of Armadillo

Wingless signal and Zeste-white3 kinase act antagonistically in Armadillo regulation. Wingless stabilizes intracellular Armadillo and decreases average levels of Armadillo phosphorylation, while Zeste white3 kinase destabilizes intracellular Armadillo and increases average levels of Armadillo phosphorylation (Peifer et al., 1994*a*; van Leeuwen et al., 1994). We initially suggested a model in which changes in phosphorylation were a direct consequence of Wingless signal (Peifer et al., 1994*a*). Our current data point out, however, that altered phosphorylation might only be an indirect effect of signaling, due to the changes in the relative ratios of cadherin-associated and intracellular pools of Armadillo that occur. Membrane-associated Armadillo is more highly phosphorylated than intracellular Armadillo (Peifer et al., 1994*a*). One possibility is that the kinase that phosphorylates Armadillo is localized to junctions, and thus only junctional Armadillo is highly phosphorylated. If so, when Wingless signal or inactivation of Zeste white3 raises levels of intracellular Armadillo relative to those of junctional Armadillo, the overall average levels of Armadillo phosphorylation will fall. This

model is consistent with our new data. Armadillo mutant proteins with deletions in the central repeat region (S5, S11, and S15) are less strongly associated with adherens junctions and exhibit instead abnormally high accumulation in embryonic nuclei. All three mutants are also hypophosphorylated, in proportion to their ability to assemble into junctions, consistent with the idea that junctional localization is required for phosphorylation.

We are very grateful to M. Takeichi and T. Uemura for sharing reagents critical to the success of these experiments and for sharing unpublished data. We are also grateful to B. Gumbiner, P. Polakis, and U. Tepass for sharing unpublished data; J. McCary for help in analyzing mutants; to A. Comer for help in myc-tagging technology; to S. Blair for advice on antibody staining; to B. Errede for the 9E10 cell line; to S. Whitfield for artistic and photographic help; and to J. Shields for keeping the lab running. We would like to thank members of the Peifer lab for valuable discussions and J. Adam, A. Bejsovec, J. Dangl, K. Daniels, S. Grant, G. Maroni, J. Reed, J. Thorn, and C. Waterman-Storer for helpful comments on the manuscript.

This work was supported by grants to M. Peifer from the National Institutes of Health (GM47857) and the Searle Scholars Program.

Received for publication 1 May 1996 and in revised form 26 June 1996.

References

Aberle, H., S. Butz, J. Stappert, H. Weissig, R. Kemler, and H. Hoschuetzky. 1994. Assembly of the cadherin-catenin complex in vitro with recombinant proteins. *J. Cell Sci.* 107:3655–3663.

Aberle, H., H. Schwartz, H. Hoschuetzky, and R. Kemler. 1996. Single amino acid substitutions in proteins of the *armadillo* gene family abolish their binding to α-catenin. *J. Biol. Chem.* 271:1520–1526.

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

Blair, S.S. 1993. Mechanisms of compartment formation: evidence that nonproliferating cells do not play a critical role in defining the D/V lineage restriction in the developing wing. *Development (Camb.).* 119:339–351.

Chou, T.-B., and N. Perrimon. 1992. Use of a yeast site-specific recombinase to produce female germline chimeras in *Drosophila*. *Genetics.* 131:643–653.

Cox, R.T., C. Kirkpatrick, and M. Peifer. 1996. Armadillo is required for adherens junction assembly, cell polarity, and morphogenesis during *Drosophila* embryogenesis. *J. Cell Biol.* 134:133–148.

Evan, G.I., G. Lewis, G. Ramsay, and J.M. Bishop. 1985. Isolation of monoclonal antibodies specific for human c-myc proto-oncogene product. *Mol. Cell. Biol.* 5:3610–3616.

Fagatto, F., N. Funayama, U. Glück, and B.M. Gumbiner. 1996. Binding to cadherins antagonizes the signaling activity of β-catenin during axis formation in Xenopus. *J. Cell Biol.* 132:1105–1114

Funayama, N., F. Fagatto, P. McCrea, and B.M. Gumbiner. 1995. Embryonic axis induction by the Armadillo repeat domain of β-catenin: evidence for intracellular signaling. *J. Cell Biol.* 128:959–968.

Haegel, H., L. Larue, M. Ohsugi, L. Federov, K. Herrenknecht, and R. Kemler. 1995. Lack of β-catenin affects mouse development at gastrulation. *Development (Camb.).* 121:3529–3537.

Hamaguchi, M., N. Matsuyoshi, Y. Ohnishi, B. Gotoh, M. Takeichi, and Y. Nagai. 1993. $p60^{v\text{-}src}$ causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:307–314.

Heasman, J., A. Crawford, K. Goldstone, P. Garner-Hamrick, B. Gumbiner, P. McCrea, C. Kintner, C.Y. Noro, and C. Wylie. 1994. Overexpression of cadherins and underexpression of β-catenin inhibit dorsal mesoderm induction in early *Xenopus* embryos. *Cell.* 79:791–803.

Herrenknecht, K., M. Ozawa, C. Eckerskorn, F. Lottspeich, and R. Kemler. 1991. The uvomorulin-anchorage protein α-catenin is a vinculin homologue. *Proc. Natl. Acad. Sci. USA.* 88:9156–9160.

Hinck, L., I.S. Näthke, J. Papkoff, and W.J. Nelson. 1994. β-Catenin: a common target for the regulation of cell adhesion by the Wnt-1 and Src signaling pathways. *Trends Biochem. Sci.* 19:538–542.

Hirano, S., N. Kimoto, Y. Shimoyama, S. Hirohashi, and M. Takeichi. 1992. Identification of a neural α-catenin as a key regulator of cadherin function and multicellular organization. *Cell.* 70:293–301.

Hoschuetzky, H., H. Aberle, and R. Kemler. 1994. β-Catenin mediates the interaction of the cadherin-catenin complex with epidermal growth factor receptor. *J. Cell Biol.* 127:1375–1380.

Hülsken, J., W. Birchmeier, and J. Behrens. 1994. E-cadherin and APC compete for the interaction with β-catenin and the cytoskeleton. *J. Cell Biol.* 127: 2061–2069.

Jou, T., D.B. Stewart, J. Stappert, W.J. Nelson, and J.A. Marrs. 1995. Genetic and biochemical dissection of protein linkages in the cadherin-catenin complex. *J. Cell Biol.* 92:5067–5071.

Karnovsky, A., and M.W. Klymkowsky. 1995. Anterior axis duplication in Xenopus induced by the over-expression of the cadherin-binding protein plakoglobin. *Proc. Natl. Acad. Sci. USA.* 92:4522–4526.

Kemler, R. 1993. From cadherins to catenins: cytoplasmic protein interactions and regulation of cell adhesion. *Trends Genet.* 9:317–321.

Kirkpatrick, C., and M. Peifer. 1995. Not just glue: cell-cell junctions as cellular signaling centers. *Curr. Opin. Genet. Dev.* 5:56–65.

Klemenz, R., U. Weber, and W.J. Gehring. 1987. The *white* gene as a marker in a new P-element vector for gene transfer in *Drosophila*. *Nucleic Acids Res.* 15:3947–3959.

Klymkowsky, M.W., and B. Parr. 1995. The body language of cells: the intimate connection between cell adhesion and behavior. *Cell.* 83:5–8.

Larue, L., M. Ohsugi, J. Hirchenhain, and R. Kemler. 1994. E-cadherin null mutant embryos fail to form a trophectoderm epithelium. *Proc. Natl. Acad. Sci. USA.* 91:8263–8267.

McCrea, P.D., W.M. Brieher, and B.M. Gumbiner. 1993. Induction of a secondary body axis in *Xenopus* by antibodies to β-catenin. *J. Cell Biol.* 123:477–484.

Munemitsu, S., B. Souza, I. Albert, B. Rubinfeld, and P. Polakis. 1995. Regulation of intracellular β-catenin levels by the adenomatous polyposis coli (APC) tumor suppressor protein. *Proc. Nat. Acad. Sci. USA.* 92:3046–3050.

Nagafuchi, A., M. Takeichi, and S. Tsukita. 1991. The 102 kD cadherin-associated protein: similarity to vinculin and post-transcriptional regulation of expression. *Cell.* 65:849–857.

Oda, H., T. Uemura, K. Shiomi, A. Nagafuchi, S. Tsukita, and M. Takeichi. 1993. Identification of a *Drosophila* homologue of alpha-catenin and its association with *armadillo* protein. *J. Cell Biol.* 121:1133–1140.

Oda, H., T. Uemura, Y. Harada, Y. Iwai, and M. Takeichi. 1994. A *Drosophila* homolog of cadherin associated with Armadillo and essential for embryonic cell-cell adhesion. *Dev. Biol.* 165:716–726.

Oyama, T., Y. Kanai, A. Ochiai, S. Akimoto, T. Oda, K. Yanagihara, A. Nagafuchi, S. Tsukita, S. Shibamoto, F. Ito, et al. 1994. A truncated β-catenin disrupts the interaction between E-cadherin and α-catenin: a cause of loss of intracellular adhesiveness in human cancer cell lines. *Cancer Res.* 54:6282–6287.

Parr, B.A., and A.P. McMahon. 1994. *Wnt* genes and vertebrate development. *Curr. Opin. Genet. Dev.* 4:523–528.

Peifer, M. 1993. The product of the *Drosophila* segment polarity gene *armadillo* is part of a multi-protein complex resembling the vertebrate adherens junction. *J. Cell Sci.* 105:993–1000.

Peifer, M. 1995. Cell adhesion and signal transduction: the Armadillo connection. *Trends Cell Biol.* 5:224–229.

Peifer, M., and E. Wieschaus. 1990. The segment polarity gene *armadillo* encodes a functionally modular protein that is the *Drosophila* homolog of human plakoglobin. *Cell.* 63:1167–1178.

Peifer, M., and E. Wieschaus. 1993. The *Drosophila melanogaster* segment polarity gene *armadillo* is highly conserved in sequence and expression in the house fly, *Musca domestica*. *J. Mol. Evol.* 36:224–233.

Peifer, M., C. Rauskolb, M. Williams, B. Riggleman, and E. Wieschaus. 1991. The segment polarity gene *armadillo* affects the *wingless* signaling pathway in both embryonic and adult pattern formation. *Development (Camb.).* 111: 1028–1043.

Peifer, M., S. Orsulic, D. Sweeton, and E. Wieschaus. 1993. A role for the *Drosophila* segment polarity gene *armadillo* in cell adhesion and cytoskeletal integrity during oogenesis. *Development (Camb.).* 118:1191–1207.

Peifer, M., L.-M. Pai, and M. Casey. 1994*a*. Phosphorylation of the *Drosophila* adherens junction protein Armadillo: roles for Wingless signal and Zeste white-3 kinase. *Dev. Biol.* 166:543–556.

Peifer, M., D. Sweeton, M. Casey, and E. Wieschaus. 1994*b*. *wingless* signal and Zeste-white 3 kinase trigger opposing changes in the intracellular distribution of Armadillo. *Development (Camb.).* 120:369–380.

Peifer, M., S. Berg, and A.B. Reynolds. 1994*c*. A repeating amino acid motif shared by proteins with diverse cellular roles. *Cell.* 76:789–791.

Riethmacher, D., V. Brinkmann, and C. Birchmeier. 1995. A targeted mutation in the mouse E-cadherin results in defective preimplantation development. *Genetics.* 92:855–859.

Riggleman, B., E. Wieschaus, and P. Schedl. 1989. Molecular analysis of the *armadillo* locus: uniformly distributed transcripts and a protein with novel internal repeats are associated with a *Drosophila* segment polarity gene. *Genes Dev.* 3:96–113.

Riggleman, B., P. Schedl, and E. Wieschaus. 1990. Spatial expression of the *Drosophila* segment polarity gene *armadillo* is post-transcriptionally regulated by *wingless*. *Cell.* 63:549–560.

Rimm, D.L., E.R. Koslov, P. Kebriaei, C.D. Cianci, and J.S. Morrow. 1995. Alpha 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proc. Natl. Acad. Sci. USA.* 92:8813–8817.

Rubinfeld, B., B. Souza, I. Albert, O. Muller, S.H. Chamberlain, F.R. Masiarz, S. Munemitsu, and P. Polakis. 1993. The APC gene product associates with β-catenin. *Science (Wash. DC).* 262:1731–1734.

Rubinfeld, B., B. Souza, I. Albert, S. Munemitsu, and P. Polakis. 1995. The APC protein and E-cadherin form similar but independent complexes with α-catenin, β-catenin, and plakoglobin. *J. Biol. Chem.* 270:5549–5555.

Rubinfeld, B., I. Albert, E. Profiri, C. Fiol, S. Munemitsu, and P. Polakis. 1996.

Association of GSK-β with the APC/β-catenin complex and regulation of complex assembly. *Science (Wash. DC).* 272:1023–1026.

Sacco, P.A., T.M. McGranahan, M.J. Wheelock, and K.R. Johnson. 1995. Identification of plakoglobin domains required for association with N-cadherin and α-catenin. *J. Biol. Chem.* 270:20201–20206.

Spradling, A.C. 1986. P element-mediated transformation. *In* Drosophila, a Practical Approach. D.B. Roberts, editor. IRL Press, Oxford, England. 175–198.

Su, L.-K., B. Vogelstein, and K.W. Kinzler. 1993. The APC tumor suppressor protein associates with catenins. *Science (Wash. DC).* 262:1734–1737.

Tepass, U., E. Gruszynski-DeFeo, T.A. Haag, L. Omatyar, T. Török, and V. Hartenstein. 1996. *shotgun* encodes *Drosophila* E-cadherin and is preferentially required during cell rearrangement in the neurectoderm and other morphogenetically active epithelia. *Genes Dev.* 10:672–685.

Uemura, T., H. Oda, R. Kraut, S. Hatashi, Y. Kataoka, and M. Takeichi. 1996. Zygotic *D* E-cadherin expression is required for the processes of dynamic epithelial cell rearrangement in the *Drosophila* embryo. *Genes Dev.* 10:659–671.

van Leeuwen, F., C. Harryman-Samos, and R. Nusse. 1994. Biological activity of soluble wingless protein in cultured *Drosophila* cells. *Nature (Lond.).* 368: 342–344.

Wieschaus, E., and C. Nüsslein-Volhard. 1986. Looking at embryos. *In* Drosophila, A Practical Approach. D.B. Roberts, editor. IRL Press, Oxford, England. 199–228.

Wieschaus, E., C. Nüsslein-Volhard, and G. Jürgens. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*: zygotic loci on the X-chromosome and the fourth chromosome. *Roux's Arch. Dev. Biol.* 193:296–307.