

# Armadillo Is Required for Adherens Junction Assembly, Cell Polarity, and Morphogenesis during *Drosophila* Embryogenesis

Rachel T. Cox,\* Catherine Kirkpatrick,<sup>‡</sup> and Mark Peifer\*<sup>‡</sup>

\*Curriculum in Genetics and Molecular Biology, <sup>‡</sup>Department of Biology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-3280

**Abstract.** Morphological and biochemical analyses have identified a set of proteins which together form a structure known as the adherens junction. Elegant experiments in tissue culture support the idea that adherens junctions play a key role in cell–cell adhesion and in organizing cells into epithelia. During normal embryonic development, cells quickly organize epithelia; these epithelial cells participate in many of the key morphogenetic movements of gastrulation. This prompted the hypothesis that adherens junctions ought to be critical for normal embryonic development. *Drosophila* Armadillo, the homologue of vertebrate  $\beta$ -catenin, is a core component of the adherens junction protein complex and has been hypothesized to be es-

sential for adherens junction function in vivo. We have used an intermediate mutant allele of *armadillo*, *armadillo*<sup>XP33</sup>, to test these hypotheses in *Drosophila* embryos. Adherens junctions cannot assemble in the absence of Armadillo, leading to dramatic defects in cell–cell adhesion. The epithelial cells of the embryo lose adhesion to each other, round up, and apparently become mesenchymal. Mutant cells also lose their normal cell polarity. These disruptions in the integrity of epithelia block the appropriate morphogenetic movements of gastrulation. These results provide the first demonstration of the effect of loss of adherens junctions on *Drosophila* embryonic development.

CELL adhesion is critically important for the development of multicellular animals as well as for the integrity of their tissues. Cells must recognize their neighbors and respond accordingly with changes in shape or molecular makeup. Cell–cell contacts are thought to be particularly important during embryonic development, when the complex morphogenetic movements of gastrulation and neurulation require cells to remain in contact, often as part of organized epithelia, while retaining the ability to move with respect to their neighbors (for review see Gumbiner, 1992). Cell–cell contacts are not only important for normal development, but are also disrupted in certain diseases such as metastatic cancer. Tumor cells have lost their ability to adhere normally to surrounding cells and instead migrate to new locations in the affected organism. Some or all of these cells' adhesive junctions must be altered or abolished; this has been recently confirmed by molecular analysis (for reviews see Takeichi, 1993; Behrens, 1994; Birchmeier, 1995).

Epithelial tissues consist of tightly juxtaposed cells forming a polarized sheet with differing apical and basal surfaces. Cell–cell adhesion between epithelial cells is me-

diated by specific adhesive junctions, the most universal of which is the adherens junction or zonula adherens (for review see Kemler, 1993). Adherens junctions allow cells (1) to assemble themselves into epithelial and nonepithelial tissues, (2) to establish and maintain cell polarity, (3) to maintain cell shape by organizing the actin cytoskeleton, and (4) to localize certain cell–cell signaling pathways. The adherens junction forms a belt around the apical end of epithelial cells; adherens junctions assembled from analogous components are found in most, if not all, multicellular animals.

Vertebrate adherens junction components include the transmembrane protein E-cadherin, which is associated with at least four cytoplasmic catenins:  $\alpha$ -catenin,  $\beta$ -catenin, plakoglobin, and p120 (Kemler, 1993; Reynolds et al., 1994; Shibamoto et al., 1995; Aghib and McCrea, 1995; Staddon et al., 1995). The fruit fly has essentially identical adherens junctions, with homologues of E-cadherin (DE-cadherin; Oda et al., 1994),  $\alpha$ -catenin (D- $\alpha$ -catenin; Oda et al., 1993), and  $\beta$ -catenin (Armadillo; Peifer, 1993; no plakoglobin homolog has been identified).  $\alpha$ -Catenin, a distant vinculin relative (Nagafuchi et al., 1991; Herrenknecht et al., 1991), may mediate association with actin.  $\beta$ -Catenin/Armadillo links cadherin and  $\alpha$ -catenin, and possibly serves as a point at which complex assembly and disassembly are regulated. Less is known about p120; phosphorylation of this Src substrate may regulate the

Address correspondence to Mark 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

properties of cell–cell junctions (Kinch et al., 1995). Studies using mammalian cell lines have demonstrated that E-cadherin (Nagafuchi et al., 1987),  $\beta$ -catenin (Kawanishi et al., 1995; Oyama et al., 1994), and  $\alpha$ -catenin (Hirano et al., 1992) are all necessary for maintaining cell–cell adhesion.  $\alpha$ -Catenin was also shown to be required for assembly of epithelia in vitro.

In addition to examining the properties of adherens junctions in tissue culture, it is important to test their function in intact animals during normal development, in both epithelial and nonepithelial tissues. We previously examined development of nonepithelial *Drosophila* ovarian germ-line cells in the absence of Armadillo, the  $\beta$ -catenin homologue. In those cells, Armadillo and thus adherens junctions are essential for normal cell function; in Armadillo's absence, cells lose their characteristic shapes, no longer adhere to their neighbors, and have disorganized actin cytoskeletons (Peifer et al., 1993). These disruptions are consistent with the idea that adherens junctions regulate cell adhesion and anchor the actin cytoskeleton. Recently, examination of *Drosophila* embryos depleted for DE-cadherin also revealed phenotypes consistent with alterations in epithelial cell organization (Uemura et al., 1996; Tepass et al., 1996). Mice missing either E-cadherin (Larue et al., 1994; Riethmacher et al., 1995) or  $\beta$ -catenin (Haegel et al., 1995) have been generated and the resulting phenotypes analyzed. E-cadherin mutant embryos have severe defects in pre-implantation stages; cells of the morula disassociate. Compaction, a process thought to require E-cadherin, is normal, presumably due to maternally provided E-cadherin.  $\beta$ -Catenin mutant embryos progress further, possibly due to a greater maternal contribution of  $\beta$ -catenin relative to E-cadherin. They implant and develop to the egg-cylinder stage. However, as gastrulation commences, the cells of the inner cell mass lose adhesion to each other preventing further development. These experiments provide dramatic confirmation of the role of adherens junction components in normal embryogenesis. However, the remaining maternal contribution of these two proteins and the presence in mice of other cadherins and of plakoglobin, a  $\beta$ -catenin relative, complicate analysis.

This sort of analysis is simpler in *Drosophila*, due to (1) the availability of techniques which allow removal of maternal gene product, revealing the earliest requirements for a protein, (2) the possible absence of functionally redundant proteins at early stages in development, and (3) the fact that the earliest stages of *Drosophila* development occur in a nuclear syncytium which is essentially a single cell. Since there are no other cells with which to make contact, adherens junction proteins are not essential during this phase of development. After 13 synchronous rounds of nuclear division unaccompanied by cytokinesis, the 6,000 nuclei cellularize synchronously to create the cellular blastoderm. It is thus possible to generate a 6,000 cell epithelial sheet in which all cells are simultaneously deprived of the adherens junction protein, Armadillo.

*Drosophila* Armadillo and vertebrate  $\beta$ -catenin are >70% identical in amino acid sequence along their entire length (for review see Peifer, 1995). In addition to their apparent role in junctions, both Armadillo (Wieschaus et al., 1984; Peifer et al., 1994) and  $\beta$ -catenin (McCrea et al., 1993; Heasman et al., 1994; Funayama et al., 1995) play

distinct roles in transduction of the Wingless/Wnt cell–cell signal which mediates cell fate determination. Embryos that are zygotically null for *armadillo* (*arm*)<sup>1</sup> have a segment polarity phenotype (Wieschaus et al., 1984), with alterations in the normal reiterated pattern of naked cuticle and denticles on the larval ventral epidermis. These embryos do not, however, show any obvious defects in cell adhesion. The large maternal contribution of wild-type Armadillo (Wieschaus and Noell, 1986) is not sufficient for Wingless signaling (Peifer, 1995), but it may be sufficient for adherens junction function. Previous attempts to completely remove the maternal contribution were unsuccessful, as Armadillo function is also essential for oogenesis (Peifer et al., 1993).

To reveal the role of Armadillo in cell adhesion, we generated embryos in which the maternal and zygotic contribution consisted of protein encoded by the mutant allele *arm*<sup>XP33</sup>, an intermediate *arm* allele (Peifer and Wieschaus, 1990). In females whose germ lines are homozygous for this mutation, oogenesis occasionally succeeds, producing embryos greatly depleted for both maternal and zygotic Armadillo function. These embryos reveal the earliest requirements for Armadillo and allow us to test the hypothesis that Armadillo, and by extension adherens junctions, are essential for cell–cell adhesion and epithelial integrity during *Drosophila* embryogenesis. When Armadillo is depleted from the onset of embryogenesis, adherens junctions fail to assemble, and normal cell polarity is disrupted. As a result, striking defects in cell–cell adhesion are observed. Rather than forming an organized epithelium, mutant cells round up and disassociate, forming a multi-layered mesenchyme. This disruption of cell–cell adhesion provides the first demonstration that Armadillo is required for adherens junction function during embryogenesis. Without normal cell–cell adhesion, gastrulation and development are disrupted. These results confirm the essential role of Armadillo in the formation of adherens junctions and the integrity of epithelial tissues in *Drosophila* embryos.

## Materials and Methods

### Fly Stocks and Generation of Germ-line Clones

*arm*<sup>XP33</sup> *FRT101/FM7*, *arm*<sup>YD35</sup> *FRT101/FM7*, and *arm*<sup>H8.6</sup> *FRT101/FM7* are described in Peifer and Wieschaus (1990) (embryonic phenotypes) and Peifer et al. (1993) (germ-line clone phenotypes). To produce embryos depleted for maternal Armadillo, we produced homozygous clones of mutant cells in the female germ line using the FLP-DFS technique of Chou and Perrimon (1992) (FLP is a site-specific yeast recombinase, DFS refers to the use of the dominant female sterile mutation *F5(1) ovo*<sup>D1</sup>). The stock used to make germ-line clones, *XX y f/ovo*<sup>D1</sup> *FRT101/Y; hsf1p-F38 /hsf1p-F38* is described in Chou and Perrimon (1992). *crb*<sup>11A22</sup> and *crb*<sup>D88-3</sup> are described in Tepass and Knust (1990). The DE-cadherin deficiency, *Df* (2R) *E2*, is described in Uemura et al. (1996). *P[arm-cmyc]* is an autosomal insert of a fully functional *arm*<sup>+</sup> gene, that rescues a null *arm* mutation to adult viability and fertility. The wild-type stock used was Canton S. All other mutations or chromosomes are described in Lindsley and Zimm (1992). Germ-line clones were generated by heat-shocking second instar larvae of the genotype *arm*<sup>XP33</sup> *FRT101/ovo*<sup>D1</sup> *FRT101; hsf1p-F38/+* for 3 h at 37°C as described in Peifer et al. (1993). Because of the dominant female sterile mutation, only germ cells homozygous mutant for

1. Abbreviations used in this paper: *arm*, armadillo; f-actin, filamentous actin; *crb*, crumbs.

*arm<sup>XP33</sup>* and homozygous wild-type for *ovo<sup>D1</sup>* develop; thus, females with ovaries have only *arm<sup>XP33</sup>* homozygous mutant germ cells.

### Antibody Staining, Visualization of Actin and DNA, and Acridine Orange Staining

Eggs from females with *arm<sup>XP33</sup>* mutant germ lines were collected from apple-juice/agar plates, washed in 0.1% Triton X-100, and dechorionated with 50% bleach. Developing embryos were picked under halocarbon oil and fixed for 1 h in 1:1 4% formaldehyde/0.1 M Pipes/2 mM MgSO<sub>4</sub>/1 mM EGTA/0.1% NP-40:heptane (Peifer et al., 1993). After manual removal of the vitelline membrane, embryos stained with anti-Arm (1:200) or anti- $\alpha$ -catenin antibody (1:10) were prepared for immunofluorescence as described for ovaries in Peifer et al. (1993). For visualization of phosphotyrosine or DE-cadherin, embryos were fixed, stained, and washed in 0.1 M phosphate buffer (pH 7.2); 0.3% Triton X-100 was added for DE-cadherin. Antibodies used were rabbit polyclonal anti-Arm (Riggleman et al., 1990), rat monoclonal anti- $\alpha$ -catenin (1:10) (Oda et al., 1993), rat monoclonal anti-DE-cadherin (1:50) (Oda et al., 1994), and mouse monoclonal anti-phosphotyrosine (UBI; 1:500). A Zeiss laser scanning confocal microscope was used to examine the stained embryos and ovaries. To visualize actin and DNA, we followed the procedure of Orsulic and Peifer (1994). Embryos were collected, fixed, and devitellinized as for antibody staining. The embryos were then treated with 400  $\mu$ g/ml RNase for 2 h at room temperature, rinsed three times with PBS, and stained for 20 min using 10  $\mu$ g/ml propidium iodide and 5 U phalloidin (BODIPY FL phalloidin 503/512, Molecular Probes, Eugene, OR). Embryos were washed three times with PBS and mounted in Aquapolymount. Ovaries were prepared as described in Peifer et al. (1993). For acridine orange staining, embryos were collected at different time points, washed, dechorionated, added to 1:1 10  $\mu$ g/ml acridine orange (Sigma Chem. Co., St. Louis, MO) in 0.1 M phosphate buffer, pH 7.2:heptane and nutated for 10 min. The embryos were transferred to a slide and covered with halocarbon oil and a coverslip. Pictures were taken immediately on either a Nikon fluorescent microscope or a Zeiss laser scanning confocal microscope.

### Scanning Electron Microscopy

Embryos were collected and dechorionated as for antibody staining, and fixed in 1:1 25% glutaraldehyde:heptane for 20–30 min. Vitelline membranes were manually removed and the embryos were rinsed with PBS with 1 mg/ml BSA, incubated in 2% osmium tetroxide in PBS for 30 min, rinsed with PBS, and dehydrated through an ethanol series (50%, 70%, 90%, 100% for 15 min each). After another rinse with 100% ethanol, critical point drying was done with CO<sub>2</sub>. Dried embryos were placed on a stub with double-stick tape and shadowed with gold palladium.

### Examination of Living Embryos and Cuticle Preparations

Embryos were collected and dechorionated as for antibody staining. Cellular blastoderm-stage embryos were picked under oil and placed on a Petriperm plate (Heraeus) covered with halocarbon oil. A coverslip was gently placed on the embryos and they were photographed periodically. For cuticle preparations, after 2–3 d the coverslip was carefully removed and individual embryos were immersed in 0.1% Triton X-100. The remaining oil was removed using a pulled Pasteur pipette, and the embryos were mounted on a slide in 1:1 Hoyer's:Lactic acid and heated at 65°C for 1–3 d (Wieschaus and Nüsslein-Volhard, 1986). In the crosses examining genetic interactions between *arm* and *crb* or the cadherin deficiency, fertilized embryos were selected under oil using the dissecting microscope, percent hatching scored, and cuticle preparations made as above.

### Preparation of Embryo Extracts and Immunoblotting

Embryos were ground in SDS sample buffer and fractionated by SDS-PAGE. Immunoblots were blocked with 5% nonfat dry milk in TBS plus 0.05% Tween-20 (TBST) and incubated with 1° and 2° antibodies in the same solution. Washes were done in TBST without milk. Bound antibody was detected by ECL (Amersham Corp., Arlington Heights, IL) as recommended and blots were stripped for reprobing as described by Amersham. Antibodies used were mouse monoclonal anti-Arm at 1:500, rat monoclonal anti- $\alpha$ -catenin at 1:100 (Oda et al., 1993) and rat monoclonal anti-DE-cadherin at 1:30 (Oda et al., 1994). For Fig. 7, living *arm<sup>XP33</sup>*

*arm<sup>XP33</sup>* or *arm<sup>XP33/+</sup>* embryos were hand selected under halocarbon oil based on their phenotype.

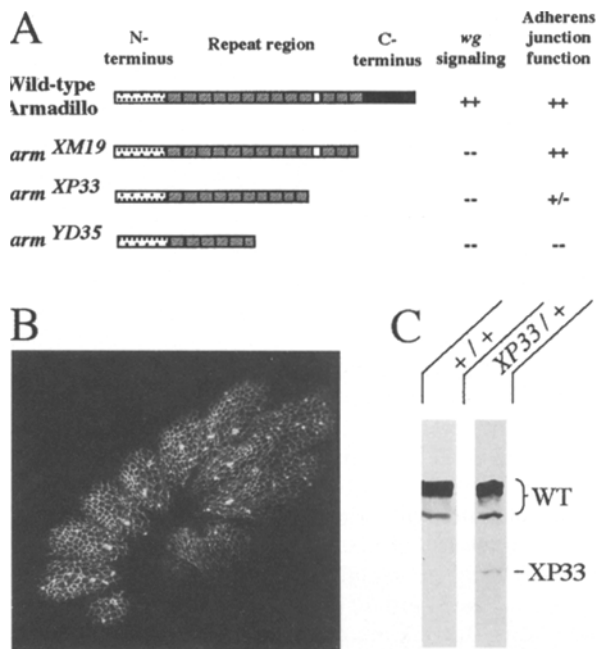
## Results

### Severely Reducing Armadillo Function Compromises Embryonic Development

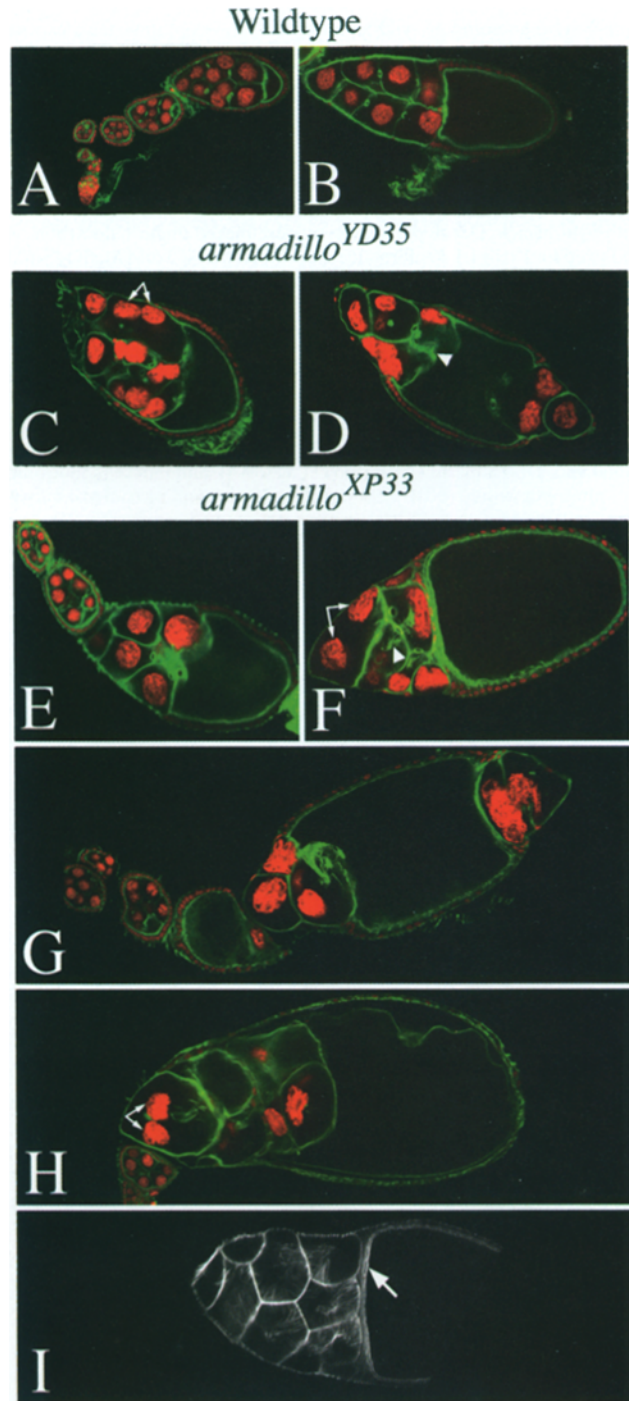
*armadillo* (*arm*) plays roles in both Wingless signal transduction and in adherens junctions (for review see Peifer, 1995). Zygotic null *arm* mutant embryos have severe segment polarity defects (Wieschaus et al., 1984; Peifer and Wieschaus, 1990), reflecting the failure of Wingless signaling, but no prominent embryonic phenotypes resulting from loss of adherens junction function are apparent (the dorsal closure defect may be a subtle result of reduction in junction function; Peifer et al., 1991). The substantial maternal contribution of Armadillo (Wieschaus and Noell, 1986) appears to be sufficient for proper junction function but not for Wingless signal transduction. Consistent with this, there is a substantial amount of remaining Armadillo in *arm<sup>YD35</sup>* zygotic null mutant embryos (Fig. 1 B).

The effects of eliminating Armadillo function on the earliest stages of embryonic development can only be seen by reducing the maternal contribution. We can generate germ-line mosaics, i.e., heterozygous mutant females in which all germ cells are homozygous mutant for *arm*—these females thus maternally contribute no Armadillo or only mutant Armadillo to their progeny. However, if the germ line is homozygous for null or severe *arm* alleles (lacking many of the Arm repeats making up the central part of the protein; Fig. 1 A), oogenesis is disrupted, resulting in defects so severe that no fertilizable eggs are laid (Wieschaus and Noell, 1986; Peifer et al., 1993). This prevents examination of mutant embryos completely lacking maternal Armadillo.

To bypass this problem and examine the requirement for Armadillo and adherens junctions early in embryogenesis, we used the intermediate *arm* allele *arm<sup>XP33</sup>*. This allele encodes a truncated protein (Fig. 1 A). *arm<sup>XP33</sup>* protein accumulates at very low levels, even compared to other mutant proteins; accumulation is <5% the level of wild type (Peifer and Wieschaus, 1990; Fig. 1 C). Ovaries in which germ-line cells are homozygous for *arm<sup>XP33</sup>* have defects similar to but less severe than those found in ovaries with germ-line cells homozygous for the null allele *arm<sup>YD35</sup>* (Fig. 2). The earliest defect seen in *arm* null mutant egg chambers is failure in adhesion between follicle and germ cells. This defect is infrequent in *arm<sup>XP33</sup>* mutant egg chambers, with the preponderance of defects at later stages. In *arm<sup>XP33</sup>* mutant egg chambers, the nurse cell cortical actin cytoskeleton is often disorganized with abnormal inclusions of actin present (Fig. 2, F and G). Perhaps as a result, membranes separating adjacent nurse cells are sometimes disrupted, yielding multinucleate nurse cells (Fig. 2, F and H). Centripetal follicle cells frequently fail to migrate and separate the oocyte from the nurse cells (Fig. 2 E), and nurse cells often fail to transfer their contents into the oocyte (Fig. 2 H). However, at a low frequency, normal egg chambers are seen (Fig. 2 I). *arm<sup>XP33</sup>* germ-line mosaic females lay fertilizable eggs, unlike germ-line mosaics for the null allele, but their egg production is greatly



**Figure 1.** Introduction. (A) Wild-type Armadillo and the proteins made by several mutant alleles. Indicated on the right is the ability of the protein to function in each of Armadillo's two roles: transduction of Wingless signal and adherens junction function. Wild-type Armadillo consists of thirteen imperfect Arm repeats flanked by NH<sub>2</sub>- and COOH-terminal regions. The COOH terminus is required for transduction of Wingless signal, while Arm repeats are important for adherens junction function (they may also function in Wingless signaling). *arm<sup>XP33</sup>* protein is intermediate in length and degree of junction function to *arm<sup>XM19</sup>* and the null allele *arm<sup>YD35</sup>*. (B) Maternally contributed Armadillo can carry out Armadillo's role in junctions. Surface view of an *arm<sup>YD35</sup>/Y* embryo stained with anti-Armadillo antibody. There is no zygotically contributed Armadillo present. The remaining staining is residual wild-type maternal Armadillo still localizing to the membrane at stage 13 of embryogenesis. (C) Protein made by *arm<sup>XP33</sup>* is truncated and present at lower levels than wild type. Embryo extract from progeny of wild-type or heterozygous *arm<sup>XP33</sup>/+* animals was fractionated by SDS-PAGE and immunoblotted with anti-Armadillo antibody. The upper bands represent differentially phosphorylated forms of wild-type Armadillo. *arm<sup>XP33</sup>* protein is present at <5% the level of wild-type protein.



**Figure 2.** *arm<sup>XP33</sup>* germ-line clones have defects during later stages of oogenesis that are similar to but less severe than those of the null allele. All egg chambers are stained with FITC-phalloidin (green) which stains filamentous actin and propidium iodide (red) which stains DNA. (A and B) Wild-type egg chambers. The oocyte is posterior to the nurse cells, and nurse cells contain single nuclei and are regularly shaped. (C and D) *arm<sup>YD35</sup>* germ-line clones, showing the null phenotype. In C, nurse cells are multinucleate (arrows) and irregularly shaped, most likely due to defects in the actin cytoskeleton. Centripetal follicle cells (arrow) could not migrate inward to enclose the oocyte. In D, the oocyte is incorrectly located in the middle of the egg chamber—multinucleate nurse cells are seen, as are actin inclusions (arrowhead).

(E–I) *arm<sup>XP33</sup>* germ-line clones. Most of the defects are milder compared to *arm<sup>YD35</sup>* germ-line clones. (E) The nurse cells often bulge out into the oocyte; in such egg chambers the centripetal cells will not migrate in to separate the oocyte and nurse cells. (F) At times the cells have migrated properly, but the nurse cells are multinucleate (arrows) and abnormal actin inclusions are seen (arrowhead). More severe phenotypes similar to those of the *arm<sup>YD35</sup>* embryos are present at a lower frequency. (G) The oocyte is occasionally improperly positioned in the middle of the egg chamber. (H) Frequently, the nurse cells fail to dump their contents into the oocyte as indicated by the large nurse cells at a late stage in oogenesis. However, normal egg chambers are present in small numbers and are able to undergo even sensitive stages of oogenesis like making the network of actin filaments which appear at the beginning of nurse cell dumping (I).

reduced compared with wild-type. Of the eggs laid, 23% are flat or half-filled, probably reflecting the observed problems during oogenesis. Of the full eggs, 94% have very lightly colored yolk and do not develop. These appear to be unfertilized, perhaps due to defects in the anterior micropyle, the eggshell structure that allows sperm entry.

About 6% of the full eggs are fertilized and begin embryonic development (Table I). We initially examined the end of embryogenesis by looking at the cuticle phenotype. Developing embryos were picked at the blastoderm stage to eliminate unfertilized eggs, and after 24 h their cuticles were prepared. Three distinct cuticle phenotypes were observed (Table I; Fig. 3). About half of the developing embryos arrest before secreting cuticle, leaving an empty vitelline membrane after cuticle preparation ("no cuticle" class). 25% of the developing embryos hatch or have a nearly normal cuticle ("wild-type" class). The remaining 25% have a more dramatic cuticle phenotype, secreting only a few cuticle scraps with no large contiguous pieces ("crumbs-like" class). This is reminiscent of but more severe than the cuticle phenotype of *crumbs* class mutants; the best characterized of these, *crumbs* (*crb*), encodes a protein normally required for establishing and maintaining epithelial cell polarity (Tepass et al., 1990; Tepass and Knust, 1990; Wodarz et al., 1993). Interestingly, a similar cuticular phenotype results from depletion of maternal and zygotic DE-cadherin (Tepass et al., 1996).

We were surprised to find three phenotypic classes in the above experiment, since there are only two genotypes. We thus manipulated the zygotic contribution, to determine which genotype yields which phenotype. *arm* is located on the X chromosome. All embryos in this experiment have only *arm*<sup>XP33</sup> contributed maternally and zygotically from their mothers. Wild-type fathers either contribute a Y chromosome, yielding embryos maternally and zygotically mutant for *arm*, or contribute a wild-type *arm*<sup>+</sup> gene.

Table I. Phenotypic analysis of eggs and embryos derived from females whose germlines are homozygous for *arm*<sup>XP33</sup>

Egg Phenotype		Embryonic Phenotype		
flat/dumple	normal	Unfertilized or pre-blastoderm arrest	Make cellular blastoderm	
23%	78%	94%	6%	
		Cuticle Phenotype		
<i>arm</i> <sup>XP33</sup> ♀ crossed to:		% "crumbs-like"	% WT	% "no cuticle"
<i>arm</i> <sup>XP33</sup> ♀				
<i>arm</i> <sup>+</sup> ♂		28	24	48
<i>arm</i> <sup>+</sup> ; <i>P</i> [ <i>arm</i> <sup>+</sup> ] ♂		0	46	54
<i>arm</i> <sup>+</sup> ♂		0	51	49
<i>Y - arm</i> <sup>+</sup> ♂				

*P*[*arm*<sup>+</sup>] = an autosomal copy of a transposon carrying a wild-type *armadillo* gene.  
*Y-arm*<sup>+</sup> = a Y chromosome, *y*<sup>2Y67g</sup>, carrying a wild-type *armadillo* gene.

Thus, our original cross yields two possible embryonic genotypes, *arm*<sup>XP33</sup>/Y and *arm*<sup>XP33</sup>/+. We altered this such that all embryos receive a zygotic *arm*<sup>+</sup> gene and have the genotype *arm*<sup>XP33</sup>/+ by mating germ line clone-carrying females with males containing the *arm*<sup>+</sup> Y chromosome duplication *y*<sup>2Y67g</sup>, or with males homozygous for an *arm*<sup>+</sup> transgene on chromosome 2 (Table I). When this is done, the crumbs-like class disappears and the number of wild-type embryos increases correspondingly (Table I). The percentage of no cuticle embryos does not change; this phenotype is thus independent of the zygotic genotype. This suggests that because of defects in oogenesis, half of the eggs are damaged so that these embryos cannot develop to secrete cuticle, regardless of their zygotic *arm* genotype. These embryos constitute the no cuticle class. The fact that the no cuticle class comprises almost exactly 50% of the embryos is apparently coincidental, and not the result of Mendelian segregation. Of the embryos developing from normal eggs, embryos that receive a zygotic *arm*<sup>+</sup> gene develop normally or nearly normally. In contrast, embryos that do not receive a zygotic *arm*<sup>+</sup> gene, such that all maternal and zygotic protein is *arm*<sup>XP33</sup> mutant, have the crumbs-like phenotype.

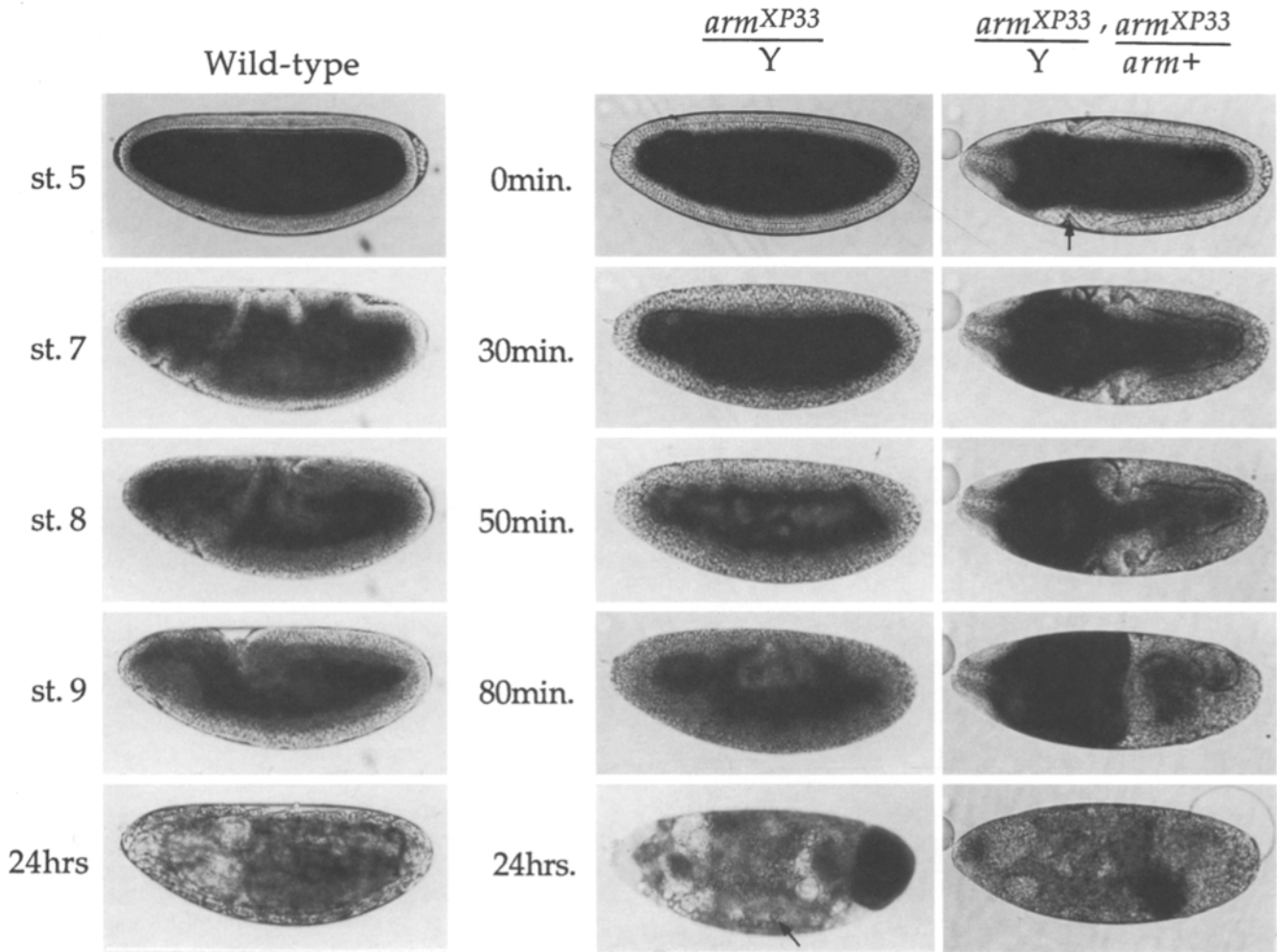
#### Embryos with Reduced Levels of Armadillo Function Show Defects at Gastrulation

Given the three possible endpoints of development observed above, we followed living embryos under the light microscope to determine when the earliest disruptions in development occurred. To correlate these observations with the cuticle phenotypes, cuticle preparations were made from all embryos observed. All three classes defined by cuticle phenotype have reproducible defects during development. We supplemented these observations by staining embryos with probes that label filamentous actin (f-actin), to outline cells and help determine cell polarity and DNA. We also observed embryos by scanning electron microscopy.

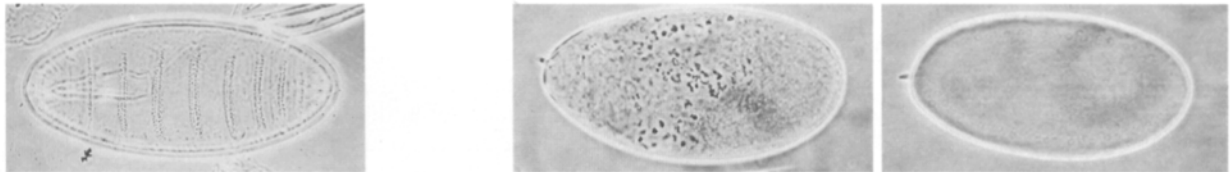
The no cuticle class of embryos deviates from normal development before the cellular blastoderm stage (Stage 5 = 0 min.; Fig. 3). They consistently fail to cellularize their anterior ends (Figs. 3 and 4) where there is a nearly complete absence of both cortical actin and nuclei (Fig. 4, A and B). The nuclei excluded from the anterior third of the egg pile up at the border between the cellularized and acellular regions, where a thick band of f-actin forms (Fig. 4, A and C); the embryos subsequently attempt to pinch off the anterior acellular yolk (Fig. 4 C). Once cellularization occurs at the egg posterior, morphogenetic movements resembling the initiation of gastrulation are sometimes observed (Fig. 3; 30–50 min.). We suspect that the cellularization defects of the no cuticle class do not reflect normal aspects of Armadillo function, but are a consequence of defects in oogenesis. The anterior cytoplasm of the egg may be altered, blocking normal syncytial development. This could be caused by defects in the anterior eggshell or yolk, due to problems with nurse cell dumping and follicle cell migration (Fig. 2).

Embryos that make wild-type cuticles exhibit wild-type or nearly wild-type embryogenesis. The syncytial blastoderm cellularizes normally (Fig. 3; stage 5), and cell move-

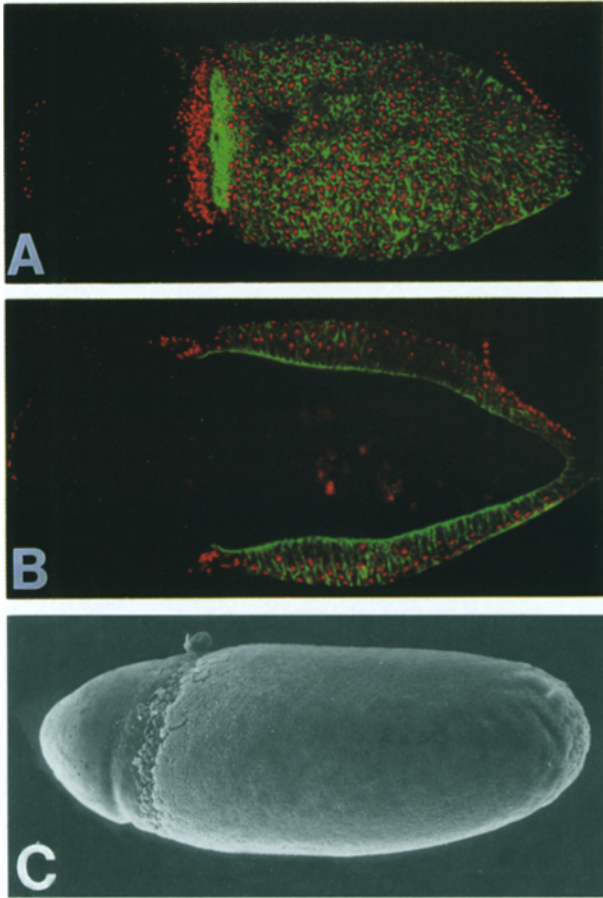
## Living Embryos



## Cuticle Phenotypes



*Figure 3.*  $arm^{XP33}$  germ-line clone-derived embryos have three cuticle phenotypes derived from three reproducible development patterns. On top are shown sequential pictures of three different living embryos (anterior at left), each representing one of the possible developmental fates seen in progeny of females with  $arm^{XP33}$  mutant germ lines. The inferred genotypes are indicated at the top. Embryos of equivalent developmental stage are shown in parallel for the three classes. On the bottom are cuticle preparations of the different classes of progeny. Wild-type embryos (on the left) have a stereotypical pattern of gastrulation and development which can be grouped into the stages indicated. After 24 h of embryogenesis, the embryo secretes cuticle with a characteristic pattern of ventral denticle belts.  $arm^{XP33}$  germ line clone-derived embryos follow three possible paths of development. One class of embryos, shown on the far right, is unable to cellularize around the entire egg circumference and does not develop far enough to secrete cuticle, leaving an empty vitelline membrane. This no cuticle class attempts to constrict off the anterior part of the embryo (arrow) which cannot cellularize. The second class of  $arm^{XP33}$  mutant embryos, shown in the middle, cellularizes normally. However, at the onset of gastrulation, the cells round up and become multilayered. Partial attempts to extend the germ band are sometimes seen. By the end of embryogenesis, this class of embryos undergoes some tissue differentiation because embryos twitch when viewed by light microscopy suggesting nerve and muscle development. In addition, balls of cells (arrow) are present which later secrete scraps of cuticle. The third class of embryos (not shown) develops like or almost like the wild-type embryos shown at the left. The wild-type stages indicated approximately the times indicated for the mutant embryos.



**Figure 4.** The no cuticle phenotype is made by embryos with severe defects in the organization of both f-actin and nuclei by the onset of cellularization. (*A* and *B*) Optical confocal sections of a no cuticle class embryo stained with FITC-phalloidin (*green*) to stain f-actin and propidium iodide (*red*) to stain DNA. In surface view (*A*), one can see that the anterior region of these embryos is unable to cellularize, perhaps due to maternal defects during oogenesis. Both nuclei and f-actin are absent in the anterior region and the missing nuclei appear to be found just anterior to a thick band of actin at  $\sim 30\%$  egg length. A cross section of the same embryo (*B*) shows that the cells that form become multilayered. (*C*) A scanning electron micrograph of a no cuticle class mutant embryo shows the embryo constricts where cellularization stops; the yolk typically bulges from the anterior end. Anterior is left in all.

ments during gastrulation are either wild-type or only marginally deviant (Fig. 3; stages 7–9). In contrast, embryos that make cuticles of the crumbs-like class (henceforth referred to as *arm*<sup>XP33</sup> mutant embryos) undergo very abnormal development. These embryos cellularize normally (Fig. 3; 0 min), but morphogenetic movements are disrupted at the onset of gastrulation (Fig. 3, 30–50 min). There is little or no germ band extension, and the ventral furrow and posterior midgut fail to invaginate normally. Some embryos make partial ventral furrows and posterior midgut invaginations that are reminiscent of normal gastrulation, while others do not make any furrows whatsoever. In all *arm*<sup>XP33</sup> mutant embryos, the epithelial monolayer of cells at the cellular blastoderm stage rapidly becomes a disordered, multi-layered mesenchyme; the

cells found in this mesenchyme round up (Fig. 3; 30–50 min).

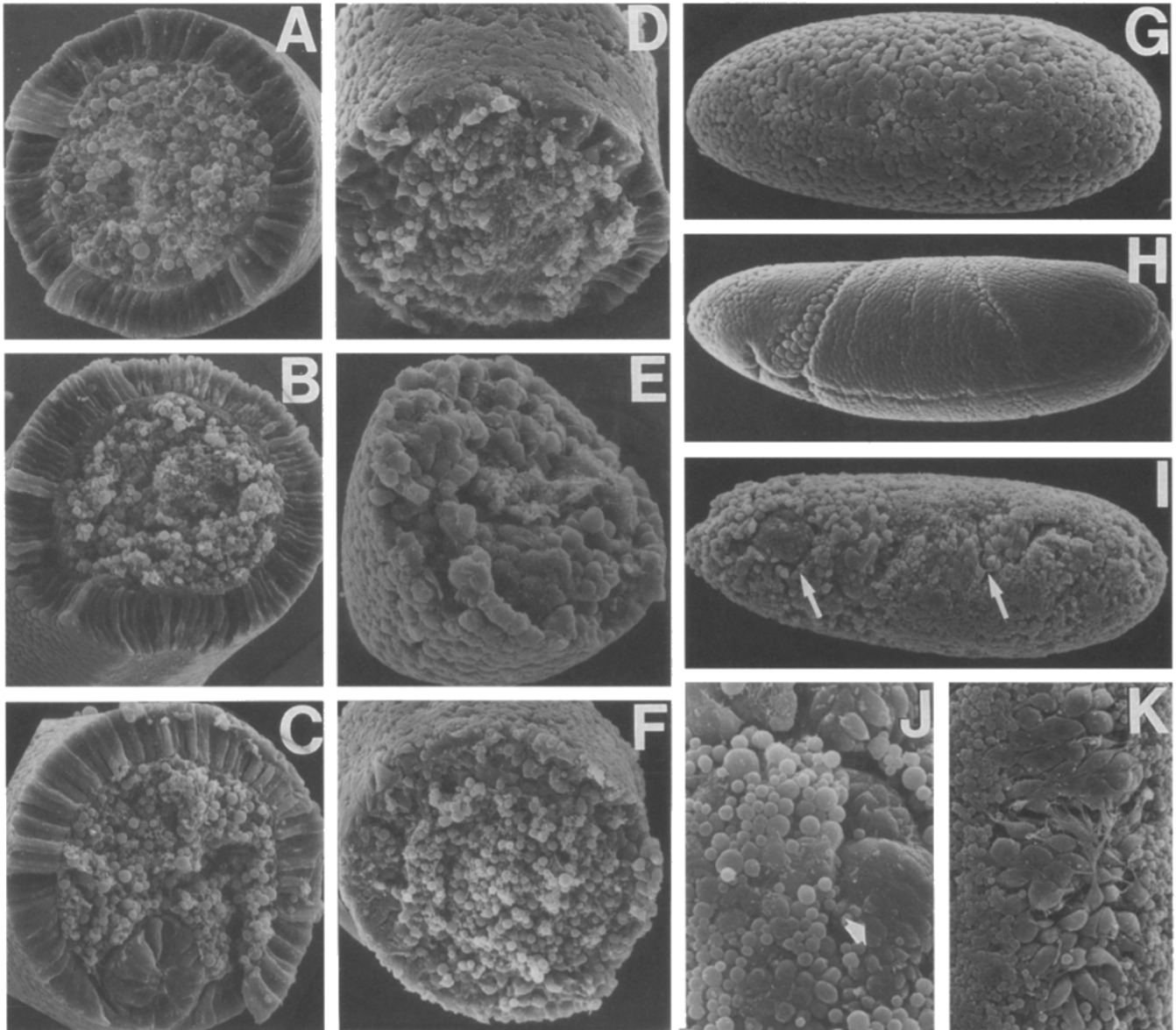
### *arm*<sup>XP33</sup> Mutants Exhibit Severely Disrupted Cell–Cell Adhesion

Scanning electron microscopy of *arm*<sup>XP33</sup> mutant embryos reveals striking defects in cell–cell adhesion. The wild-type cellular blastoderm consists of an epithelial monolayer of tightly juxtaposed columnar cells (Fig. 5, *A–C*). These cells are joined by spot adherens junctions (Tepass and Hartenstein, 1994), visible by transmission electron microscopy or by staining with antibodies to adherens junction proteins (see below). *arm*<sup>XP33</sup> mutant embryos, which are maternally and zygotically severely depleted for Armadillo, form a normal cellular blastoderm in which the cells are the proper columnar shape (Fig. 5 *A*). During cellularization, the developing cells of mutant embryos remain adherent to each other. However, as soon as cellularization is completed and gastrulation begins, defects in cell shape and organization are apparent as the cells lose adhesion to one another. At early stages, parts of the embryo remain epithelial, while in other parts cells round up and become multilayered (Fig. 5 *D*). As development proceeds, the remaining cells round up. In surface view, large, round cells are seen that appear only loosely connected (Fig. 5 *G*); in cross section many layers of round cells are seen (Fig. 5, *E* and *F*). The same changes can be observed by examining embryos stained with fluorescent markers for f-actin and nuclei (Fig. 6). The polarity of f-actin accumulation is also altered. The actin cytoskeleton of a normal ectodermal epithelial cell shows a strong polarity (Fig. 6, *A*, *B*, and *I*), in which the heaviest accumulations are seen at the apical end of the lateral surface (in particular in the adherens junction) and along the basal surface. This polarity is rapidly lost in *arm*<sup>XP33</sup> mutant embryos as cells round up and lose contact with each other; f-actin distribution becomes uniform around the entire plasma membrane, with no obvious polarity (Fig. 6, *C*, *K*, and *L*).

By  $\sim 2$  h after the onset of gastrulation, while most cells of *arm*<sup>XP33</sup> mutant embryos remain very disorganized (Fig. 5, *F* and *I*), subsets of cells recreate some types of multicellular organization (Fig. 5 *J*). Some cells cluster into balls with epithelial characteristics (Fig. 5 *J*). In addition to fully formed epithelial balls, one can see small groups of cells apparently in the process of invaginating (Fig. 6 *D*, arrowhead). Distinctive spindle-shaped cells form another type of multi-cellular aggregate (Fig. 5 *K*). Epithelial balls and spindle-shaped cells occupy distinct positions along the anterior-posterior axis, with epithelial balls most common anteriorly and spindle-shaped cells found medially. Each subset may represent distinct cell types more resistant to the disruptive effects of Armadillo depletion than their neighbors.

### Armadillo Is Required for Adherens Junction Assembly and Cell Polarity

All components of the multi-protein adherens junction complex are thought to be essential for function. We tested whether adherens junctions form in *arm*<sup>XP33</sup> mutant embryos, using antibodies to the known adherens junction components DE-cadherin,  $\alpha$ -catenin, and Armadillo (the



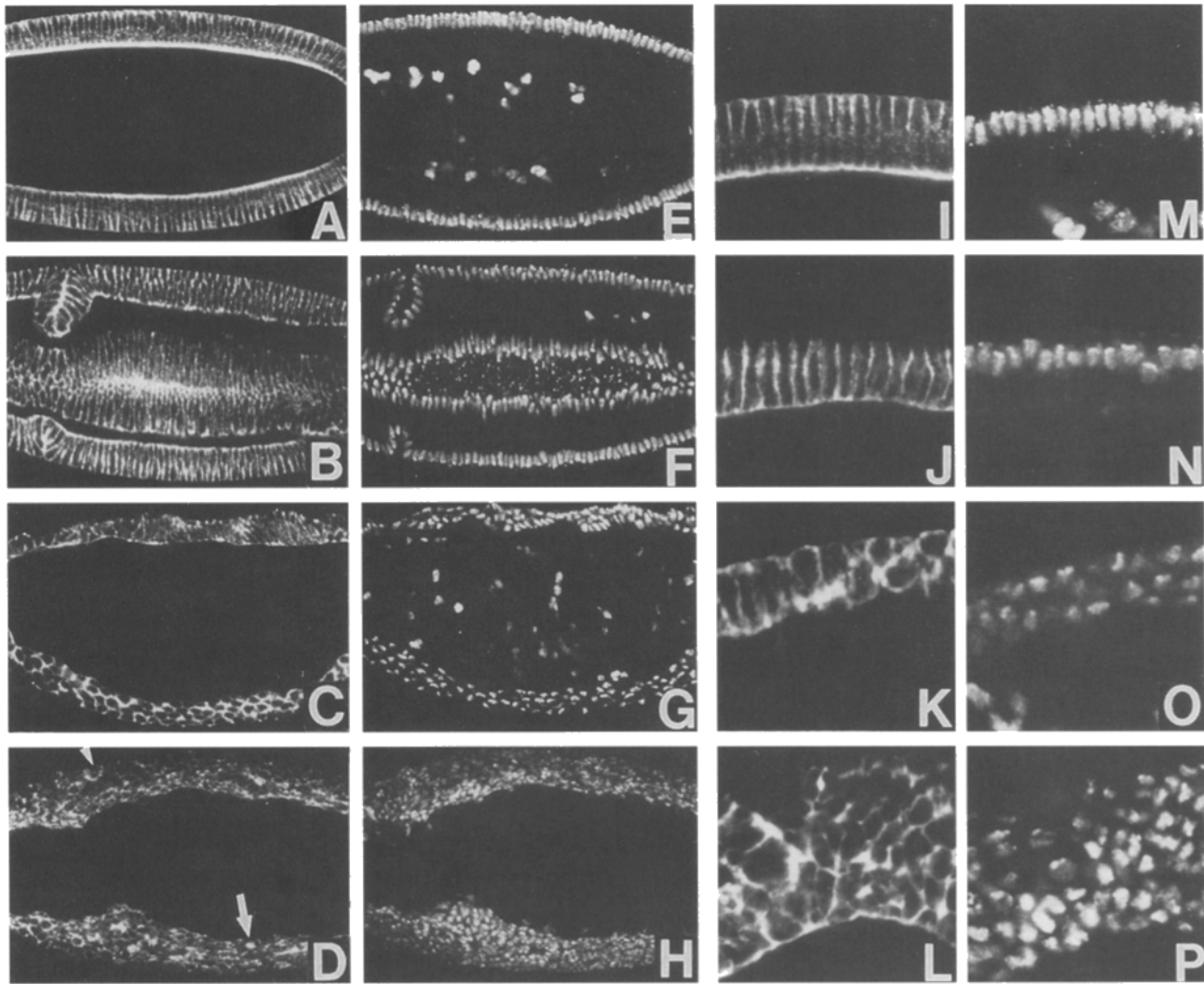
**Figure 5.** *arm<sup>XP33</sup>* mutant embryos show defects in cell-cell adhesion. All panels are scanning electron micrographs. Where it was possible to determine, anterior is left and ventral is down. (A–C) Wild-type embryos. (A) Wild-type cellular blastoderm embryos have regular, columnar cells. (B) Gastrulation is initiated when cells of the presumptive ventral furrow constrict their apices, and invaginate (C) to form a tube which later becomes mesoderm. (D–F) *arm<sup>XP33</sup>* maternally and zygotically mutant embryos. Normal gastrulation is disrupted. (D) Mutant embryos lose their columnar cells at the onset of gastrulation. At early stages, some cells (on the right) partially maintain their columnar shapes while other cells (on the left) have rounded up and become multilayered. (E) As *arm<sup>XP33</sup>* mutant embryos get older, their cells continue to become more and more multilayered as well as eventually getting smaller (F), presumably due to additional mitoses. (G and H) Surface views of *arm<sup>XP33</sup>* mutant (G) vs wild-type (H) embryos at equivalent stages. The surface of the *arm<sup>XP33</sup>* embryos (G) becomes bumpy, as cells lose contact with each other. (H) In contrast, the cells in a stage 9 wild-type embryo are tightly apposed (the round cells near the cephalic furrow are undergoing mitosis). (I–K) Later stages in the development of *arm<sup>XP33</sup>* mutant embryos. J and K represent different embryos than that seen in I. Within about 2 h after the onset of gastrulation, *arm<sup>XP33</sup>* mutant embryos have subsets of cells with distinct morphological characteristics that have reassembled into multi-cellular structures (I, arrows). These cell subsets include balls of cells that appear smooth and cohesive like epithelial cells (arrow, J) and spindle-shaped cells which extend processes (K).

truncated *arm<sup>XP33</sup>* protein reacts with anti-Armadillo antibodies that recognize the NH<sub>2</sub> terminus; Peifer et al., 1990).

We examined both the levels (Fig. 7) and the intracellular localization of junctional components (Fig. 8). Wild-type Armadillo is absent from mutants (Fig. 7 B), while

*arm<sup>XP33</sup>* mutant protein accumulates at levels substantially lower than wild type (Fig. 7 C). In contrast, both DE-cadherin and  $\alpha$ -catenin are present at levels only somewhat reduced from wild type (Fig. 7 B). These two junctional proteins are no more reduced in levels than a cytoplasmic protein, BicD, which was used as a control. The overall re-





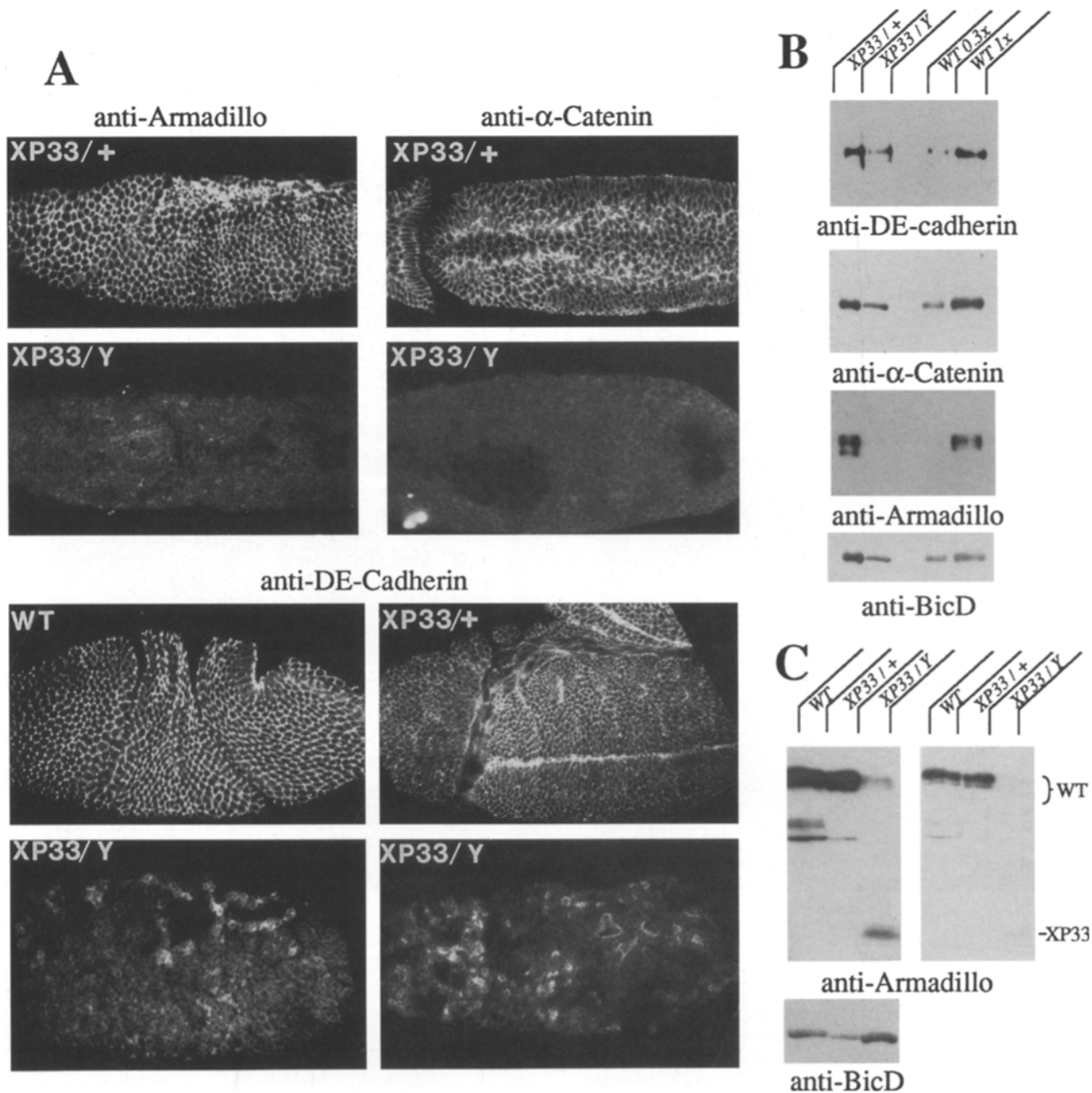
**Figure 6.** The cells of *arm<sup>XP33</sup>* mutant embryos become multilayered and lose actin cytoskeletal polarity once gastrulation begins. Embryos were doubly stained with phalloidin to stain f-actin (A–D and I–L) and propidium iodide to stain nuclei (E–H and M–P). A, B, E, F, I, and M are wild-type, while the rest are *arm<sup>XP33</sup>* mutant embryos. Adjacent pairs are images of the same embryo (e.g., A = E, B = F). (A, E, I, and M) Wild-type embryos have regular, columnar cells after cellularization, with a polarized actin cytoskeleton; actin is enriched in cell–cell adherens junction at the apical-lateral interface and in cell–matrix junctions on the basal surface. (B and F) Wild-type cells retain their columnar shape during gastrulation. (J and N) The first sign that *arm<sup>XP33</sup>* mutant embryos lose cell–cell adhesion is when cells in the cellular blastoderm lose their columnar shape, giving a bowed appearance (J) compared to wild-type (I). The corresponding nuclei, which are in a very regular array in wild-type embryos (M), slip out of place in *arm<sup>XP33</sup>* mutant embryos (N). (C, G, K, and O) Once gastrulation begins, mutant embryos quickly become multilayered (C and G) and the normal polarity of the actin cytoskeleton is lost (K and O). The cells lose their columnar shape and round up. Initially regions of the embryos remain nearly columnar next to rounded-up cells (K). These more normal regions soon disappear. (D and H) *arm<sup>XP33</sup>* mutant embryos give little or no indications of normal gastrulation. The cells become increasingly multilayered with rounded cells stacking upon each other (L and P). In later stage embryos (D), fully formed epithelial balls (arrow) and invaginating cells (arrowhead) are seen.

ductions in the level of protein per embryo may reflect a reduction in the number of cells in mutant embryos relative to wild type. The presence of Armadillo is thus not essential for continued stability of either DE-cadherin or  $\alpha$ -catenin, at least for the first few hours.

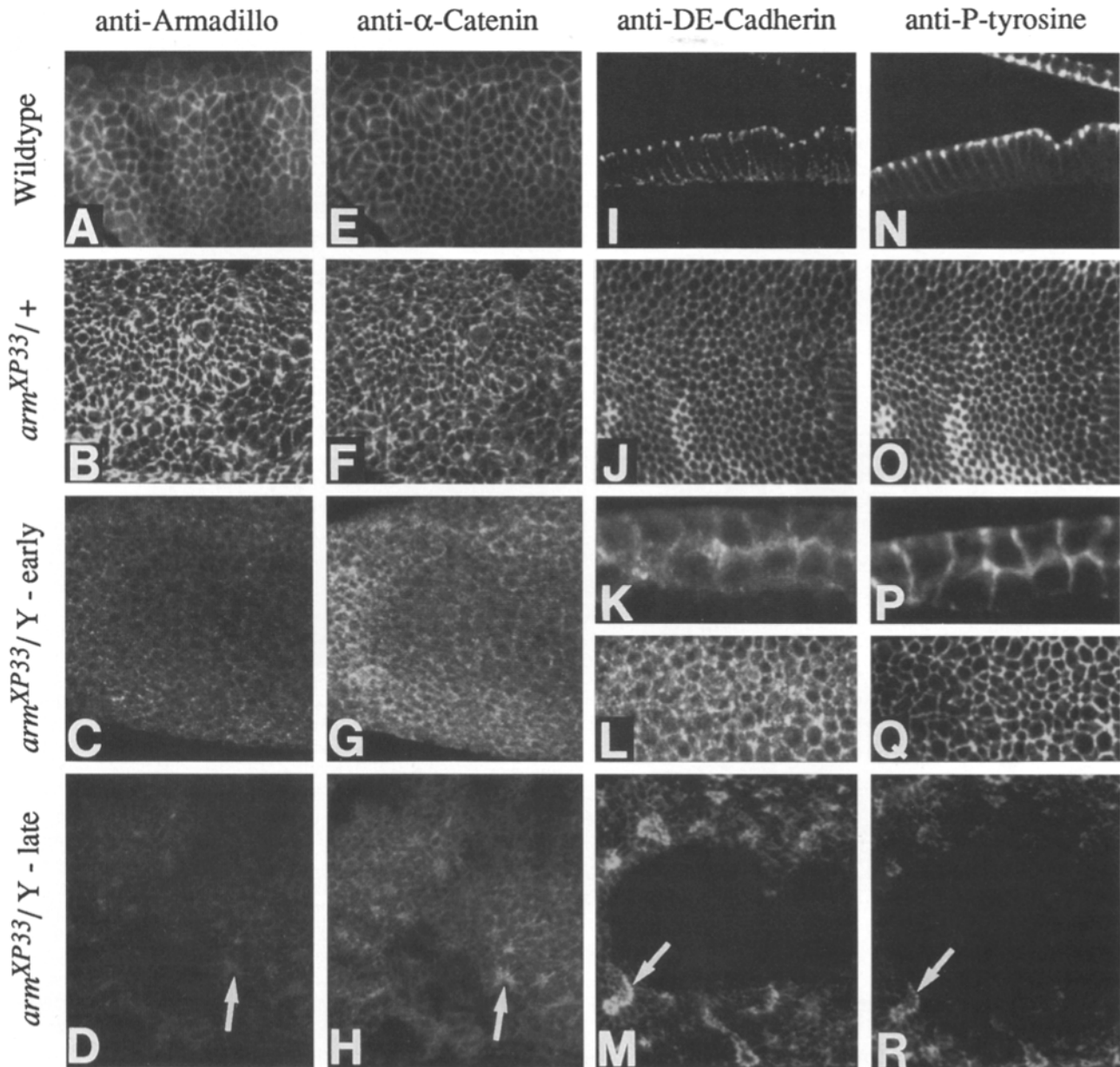
*arm<sup>XP33</sup>* mutant embryos show severely reduced levels of Armadillo staining (Fig. 8 C). The lack of Arm accumulation in maternally and zygotically mutant embryos makes them clearly distinguishable from siblings with a wild-type zygotic *arm<sup>+</sup>* gene (Fig. 8 B). We used double-label immunofluorescence to unambiguously identify *arm<sup>XP33</sup>* mutant

embryos, allowing us to examine consequences of Armadillo's absence on the intracellular localization of DE-cadherin or  $\alpha$ -catenin.

Removal of Armadillo resulted in dramatic alterations in the localization of other adherens junction proteins. In *arm<sup>XP33</sup>* mutants,  $\alpha$ -catenin no longer accumulates at the plasma membrane, but instead is found diffusely in the cytoplasm (Fig. 8 G). *arm<sup>XP33</sup>* mutant embryos also show a change in the intracellular distribution of DE-cadherin. In wild-type embryos, DE-cadherin is tightly localized to the plasma membrane and enriched in adherens junctions (Fig.



**Figure 7.** Armadillo, DE-cadherin, and  $\alpha$ -catenin levels are slightly reduced in  $arm^{XP33}$  mutant embryos. (A) Levels of Armadillo, DE-cadherin, and  $\alpha$ -catenin in  $arm^{XP33}$  mutant embryos vs heterozygous siblings. Embryos were prepared for immunofluorescence with the indicated antibodies and viewed by confocal microscopy. The same brightness and contrast settings were used for  $arm^{XP33}$  mutants and heterozygous siblings. Overall staining is reduced for all three antigens in  $arm^{XP33}$  mutants. (B) Immunoblot analysis of levels of Armadillo, DE-cadherin, and  $\alpha$ -catenin in  $arm^{XP33}$  mutants vs heterozygous siblings. We loaded equal embryo equivalents to compare protein levels per embryo, and used BicD, a cytoplasmic protein, as a control for levels of total protein. Living embryos were hand-picked under halocarbon oil as crumbs-class ( $arm^{XP33/Y}$ ) or wild-type ( $arm^{XP33/+}$ ), extracts made in SDS sample buffer, and analyzed by SDS-PAGE and sequential immunoblotting with the antibodies indicated under each blot. The left two lanes contain equal embryo equivalents of either  $arm^{XP33/+}$  or  $arm^{XP33/Y}$  extract, while the right two lanes are controls with different amounts of wild-type extract.  $arm^{XP33/+}$  embryos contain nearly as much Armadillo, DE-cadherin, and  $\alpha$ -catenin as wild-type embryos, while  $arm^{XP33/Y}$  embryos contain no wild-type Armadillo, as expected, but DE-cadherin and  $\alpha$ -catenin levels are reduced only two- to threefold; this is roughly similar to reductions in levels of total protein, indicated by BicD. (C) Levels of wild-type Armadillo vs  $arm^{XP33}$  mutant protein (the right panel is a shorter exposure). Wild-type,  $arm^{XP33/+}$  or  $arm^{XP33/Y}$  embryos were selected and treated as in B. Hand selection of embryos by phenotype is not perfect, as reflected by the low but detectable levels of wild-type Armadillo in embryos selected as  $arm^{XP33/Y}$ . Levels of wild-type Armadillo are roughly comparable between wild-type and  $arm^{XP33/+}$  embryos; zygotic expression of  $arm$  thus restores near wild-type levels even in the absence of maternal contribution, consistent with zygotic phenotypic rescue. In  $arm^{XP33/Y}$  embryos, with only  $arm^{XP33}$  mutant protein maternally and zygotically, levels of mutant protein are greater than seen in heterozygotes in Fig. 1 C, but mutant protein still accumulates at much lower levels than the wild-type protein in heterozygous siblings.



**Figure 8.** Adherens junctions fail to assemble in *arm<sup>XP33</sup>* mutant embryos, and normal cell polarity is lost. Embryos of the genotypes indicated at the left of each row were prepared for immunofluorescence with antibodies at the top of each column, and examined by confocal microscopy. (A–D) anti-Armadillo, (E–H) anti- $\alpha$ -catenin, (I–M) anti-DE-cadherin and (N–R) anti-phosphotyrosine. (A, E, I, and N) Wild-type embryos accumulate adherens junction components in a characteristic lattice pattern (A and E). In cross section (I and N), the enrichment of these proteins in the adherens junction at the apical-lateral interface can be seen. Phosphotyrosine accumulates heavily in adherens junctions. The polarized accumulation of DE-cadherin and phosphotyrosine serves as a marker of the polarity of the epithelial sheet. Anti-DE-cadherin also stains the lateral sides of cells, though not as strongly (I). (B, F, J, and O). *arm<sup>XP33</sup>/+* siblings, with a wild-type zygotic copy of *arm*, show much the same pattern as wild-type with all four markers. (C, G, K, L, P, and Q) Early *arm<sup>XP33</sup>* mutant embryos exhibit different patterns for staining than their siblings. (C) In *arm<sup>XP33</sup>* mutant embryos Armadillo is greatly reduced in level relative to their siblings; the small amount of remaining Armadillo is mostly membrane bound. (G)  $\alpha$ -Catenin faintly outlines cells and is brighter than anti-Armadillo staining, but there is a greatly increased amount of  $\alpha$ -catenin in the cytoplasm. (K and L) DE-cadherin in early *arm<sup>XP33</sup>* mutant embryos is still partially plasma membrane-associated, but there is an increased amount within cells, as part of punctate structures which may be vesicles. DE-cadherin remaining at the membrane is no longer polarized. (P and Q) Anti-phosphotyrosine staining shows the same loss of polarity but remains completely plasma membrane associated. (D, H, M, and R) In later *arm<sup>XP33</sup>* mutant embryos, epithelial balls form. Their putative apical-lateral interfaces accumulate adherens junction components. Anti-Armadillo and anti- $\alpha$ -catenin faintly stain these epithelial balls (arrows, D and H), though overall staining has decreased even more by this point. Anti-DE-cadherin and anti-phosphotyrosine antibodies brightly stain epithelial balls (arrows, M and R). The staining is polarized, accumulating in a tight dot resembling the wild-type adherens junction (I and N).

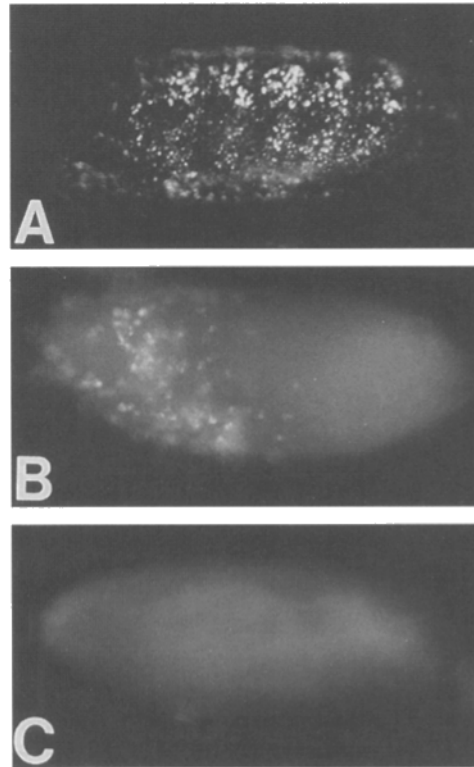
8 I). In contrast, in *arm<sup>XP33</sup>* mutant embryos DE-cadherin accumulation at the plasma membrane is reduced, and the overall staining intensity drops somewhat. Much of the remaining DE-cadherin accumulates within cells, presumably in the ER, Golgi, or endosomes (Fig. 8, K and L). This may be a result of endocytosis, which occurs in cultured mammalian cells if cell adhesion is disrupted by removal of calcium (Kartenbeck et al., 1991).

Adherens junctions are also thought to be important in the establishment of cell polarity (McNeill et al., 1990). To examine cell polarity, we used antibodies directed against DE-cadherin and phosphotyrosine. Phosphotyrosine accumulates in epithelial cells of *Drosophila* imaginal discs in a polarized fashion, with intense accumulation in the region of the adherens junction (Woods and Bryant, 1993). We have found that phosphotyrosine also serves as a polarity marker in the embryonic ectodermal epithelium; it is greatly enriched at the adherens junction (Fig. 8 N). In wild-type embryos, DE-cadherin and phosphotyrosine colocalize to adherens junctions. DE-cadherin, while apically enriched, also accumulates along the lateral cell membranes (Fig. 8, I and N).

In *arm<sup>XP33</sup>* mutant embryos, cells appear to have normal polarity during cellularization, but upon the onset of gastrulation cell polarity is rapidly disrupted. Both phosphotyrosine and DE-cadherin lose their apical enrichment and instead are found all around the circumference of the cells (Fig. 8, K and P). Phosphotyrosine continues to accumulate at the cell surface at roughly normal levels but in a delocalized fashion; however, it does remain enriched at cell-cell contact surfaces.

These disruptions in adherens junctions and cell polarity persist through the process of epithelial disintegration. However, about 2 h after the onset of gastrulation the cells in mutant embryos that join together to form epithelial balls reform apparent adherens junctions (Figs. 5 and 8). The assembly of these groups of cells can be seen using anti-phosphotyrosine antibody (Fig. 8 R, arrow). Phosphotyrosine becomes repolarized, localizing to the inside of epithelial balls at the apparent apical ends of the cells, as is seen in wild-type epithelia (Fig. 8 N). A similar repolarization of DE-cadherin is seen in *arm<sup>XP33</sup>* mutant embryos (Fig. 8 M, arrow). Armadillo and  $\alpha$ -catenin also localize to areas of epithelial organization (Fig. 8, D and H); their staining is substantially fainter, yet still is enriched in putative adherens junctions. Outside epithelial balls, the situation is quite different. At this later stage in development, many cells are labeled very poorly or not at all with all of the antibodies.

In certain systems, when cell-cell or cell-matrix interactions are disrupted, the apoptosis program is triggered (e.g., Hermiston and Gordon, 1995; Re et al., 1994). We thus examined whether the dissolution of epithelia in *arm<sup>XP33</sup>* mutant embryos led to increased or premature programmed cell death. We used acridine orange, a vital dye, to examine the pattern and timing of cell death in *arm<sup>XP33</sup>* mutant embryos. In a wild-type embryo, programmed cell death commences at stage 11,  $\sim 7$  h after egg laying, and it occurs in a very reproducible spatial and temporal pattern (Abrams et al., 1993; Fig. 9 A). In *arm<sup>XP33</sup>* mutant embryos, there was no noticeable cell death before 8 h (Fig. 9 C), and substantial cell death was



**Figure 9.** Programmed cell death occurs in an altered pattern in *arm<sup>XP33</sup>* mutant embryos. Acridine orange dye was used to visualize cells undergoing programmed cell death in *arm<sup>XP33</sup>* mutant embryos. (A) An optical section of an *arm<sup>XP33/+</sup>* embryo, showing the wild-type pattern of programmed cell death—specific cells die at specified times. Cell death normally commences at stage 11,  $\sim 7$  h after egg laying. (B) While cell death in *arm<sup>XP33</sup>* mutant embryos commences at the normal time (this embryo is greater than 12-h-old), the normal spatial pattern of programmed cell death is not seen. Instead cells die in a disorganized, seemingly random pattern. (C) Before 8 h after egg laying, *arm<sup>XP33</sup>* mutant embryos have no cells which are stained by the dye, suggesting that programmed cell death has not yet begun. (A) is a laser scanning micrograph whereas both B and C are fluorescent micrographs.

not observed until after 10 h. When cell death did occur, the spatial pattern seen in wild-type embryos was not observed, reflecting the totally altered morphology of the mutant embryos. Instead, relatively large numbers of cells died throughout the embryo (Fig. 9 B). This is similar to the timing and pattern of cell death observed in *crumbs* mutant embryos (Abrams et al., 1993).

#### **Genetic Interactions between armadillo and crumbs Are Additive**

The similarity in cuticle phenotype between *arm<sup>XP33</sup>* mutants and mutants of the *crumbs* (*crb*) class (Tepass et al., 1990; Tepass and Knust, 1990) led us to investigate genetic interactions between *arm* and *crb*. We crossed females heterozygous for both a null allele of *arm* (*arm<sup>YD35</sup>*) and a strong allele of *crb* (*crb<sup>11A22</sup>*) with males heterozygous for *crb<sup>11A22</sup>*. Among the progeny (Fig. 10 A), 3/16ths will be hemizygous for *arm<sup>YD35</sup>* and display a strong segment po-

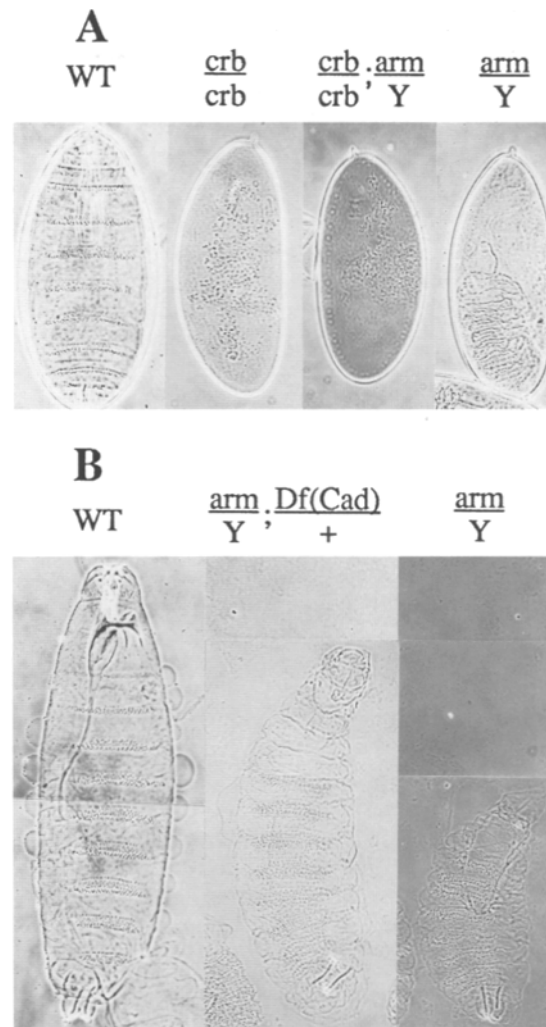
larity phenotype, 3/16ths will be homozygous for *crb*<sup>11A22</sup> and secrete many small pieces of cuticle, and 1/16th will be doubly mutant for *arm*<sup>YD35</sup> and *crb*<sup>11A22</sup>. We observed the expected *arm* and *crb* phenotypes at the expected frequencies, and also a new *crb*-like phenotype (Fig. 10 A) with considerably less cuticle than *crb*<sup>11A22</sup> homozygotes. The frequency of embryos with this severe *crb*-like phenotype suggests that they are the *arm*<sup>YD35</sup>/*Y*; *crb*<sup>11A22</sup> double mutants. This cross also generates animals heterozygous for one mutation and homozygous for the other; if any dose-sensitive interactions were to be seen, they would emerge as alterations in the phenotypes of some of the apparent single mutants. No such dose-sensitive interactions were observed. The observed double-mutant phenotype may represent the additive effect of losing epithelial integrity in an embryo which is unable to generate a full-size cuticle. A similar cross with the weak *crb*<sup>D88-3</sup> allele was also performed. Most *crb*<sup>D88-3</sup> homozygotes survive embryogenesis; those which die show occasional defects such as small cuticle holes. *arm*<sup>YD35</sup>; *crb*<sup>D88-3</sup> double mutants resembled *arm*<sup>YD35</sup> hemizygotes, but with occasional small holes in the cuticle.

### Reducing DE-Cadherin Levels Suppresses the armadillo Segment Polarity Phenotype

Given our data in support of the idea that the maternal contribution of Armadillo is normally sufficient for junction function, we were curious to determine whether reduction in the levels of another junctional component might reveal a junctional phenotype of the *arm* zygotic null. We therefore generated animals hemizygous for *arm*<sup>XP33</sup> (Fig. 10 B) and also heterozygous for a deficiency that removes the DE-cadherin gene (Fig. 10 B, *Df(2R) E2*; Uemura et al., 1996). These embryos should thus possess half as much cadherin as normal. No increase in the severity of junctional phenotypes (e.g., cuticle integrity or dorsal closure) was noted, suggesting that in this range of dosage, the system is not dose-sensitive for junction function. However, there was a substantial reduction in the severity of the segment polarity phenotype (Fig. 10 B). Animals hemizygous for *arm*<sup>YD35</sup> and heterozygous for the DE-cadherin deficiency had a similar though less dramatic suppression of their segment polarity phenotype (data not shown). We suspect that the suppression observed in both cases reflects the fact that the Armadillo's roles in adherens junctions and Wingless signaling are separable, and that under conditions where Armadillo is limiting, as in a strong hypomorphic *arm* mutant, the reduction in the number of junctional complexes frees up some of the wild-type maternal store of Armadillo protein, allowing it to function in Wingless signaling. Similar competitive interactions have been seen in *Xenopus*, where increases in cadherin levels can block action of  $\beta$ -catenin in Wnt signaling (Heasman et al., 1994).

### Discussion

Normal development requires that individual cells organize themselves into multi-cellular tissues (for review see Gumbiner, 1992). The cell-cell adherens junction or zonula adherens is thought to be a key element in the initi-



**Figure 10.** Genetic interactions between *armadillo*, *crumbs*, and *DE-cadherin*. (A) To look for interactions between *crb* and *arm*, we crossed flies doubly heterozygous for the strong alleles *crb*<sup>11A22</sup> and *arm*<sup>YD35</sup>. *crb*<sup>11A22</sup> homozygotes produce scraps of cuticle while *arm*<sup>YD35</sup> hemizygotes have a severe segment polarity defect, and they produce a cuticle much shorter than wild-type. *arm*<sup>YD35</sup> *crb*<sup>11A22</sup> double mutants secrete a reduced number of cuticle scraps relative to *crb*<sup>11A22</sup> homozygotes—the two phenotypes are roughly additive. (B) *arm*<sup>XP33</sup>/*Y* embryos have a severe segment polarity defect (though it is slightly less severe than that of the null allele *arm*<sup>YD35</sup>). *arm*<sup>XP33</sup>/*Y*; *Df(Cad)*/+ embryos, in which the amount of cadherin has been reduced by 50%, have a segment polarity defect which is substantially less severe than *arm*<sup>XP33</sup>/*Y* alone. Genotypes indicated in some cases are inferred from the frequency of a phenotype among the progeny.

ation and maintenance of epithelial organization (for review see Kemler, 1993). This theory of adherens junction function continues to be tested in tissue culture. However, testing the function of adherens junctions in intact animals has proven more challenging. By using *Drosophila* to focus on one adherens junction component, Armadillo/ $\beta$ -catenin, we can use genetic as well as biochemical and molecular approaches. We generated embryos essentially lacking Armadillo function and used these embryos to test both the role of Armadillo in adherens junctions, and the role of

adherens junctions in epithelial integrity and in morphogenesis. These mutant embryos demonstrate that Armadillo is critical for the assembly and function of adherens junctions. In the absence of Armadillo, other junctional proteins are unable to form the required complex and cannot localize properly. As a result, the cell polarity normally present in epithelial cells is lost. Without normal adherens junctions, cells fail to form organized epithelia and instead take on apparently mesenchymal character. The dissolution of the blastoderm stage epithelium blocks the normal morphological movements of gastrulation, and thus catastrophically disrupts normal embryonic development. A similar analysis of the result of depletion of Armadillo or of the products of the *bazooka* and *stardust* genes is reported in Müller and Wieschaus (1996).

### **Armadillo and Adherens Junctions Are Required for Epithelial Integrity and Gastrulation**

Scientists have used cultured mammalian cells to probe the function of adherens junction proteins (Nagafuchi et al., 1987; Hirano et al., 1992); these data support the model in which cadherin,  $\alpha$ - and  $\beta$ -catenin form an integrated machine, with each component essential for function. This model is being tested in intact animals using genetic analysis. In mice, both E-cadherin (Larue et al., 1994; Riethmacher et al., 1995) and  $\beta$ -catenin (Haegel et al., 1995) are essential for embryogenesis; mutant embryos arrest near or soon after implantation. In nonepithelial germ cells of *Drosophila*, Armadillo is required for cell architecture, adhesion, and integrity of the actin cytoskeleton. Disruptions of these properties are observed in the phenotype of germ cells homozygous for null or severely hypomorphic *arm* alleles (Peifer et al., 1993). Here we extended this analysis to an intermediate allele, *arm*<sup>XP33</sup>. This somewhat less severe allele has a similar though less severe phenotype during oogenesis—while most mutant egg chambers arrest with defects similar to those seen with the null allele, some *arm*<sup>XP33</sup> mutant egg chambers proceeded normally through oogenesis, allowing us to examine embryos severely depleted for maternally contributed Armadillo.

In this report, we describe the consequences of depletion of maternally contributed Armadillo, which otherwise partially rescues the zygotic loss of function. In *Drosophila*, the first thirteen nuclear cycles are unaccompanied by cytokinesis; 6,000 cells then form simultaneously. Junctional components are dispensable in the syncytial phase, as there is only one cell, so we can observe 6,000 cells simultaneously deprived of Armadillo. Since blastoderm cells are large, we could also examine the effects of Armadillo depletion on intracellular localization of other junctional proteins.

The results of this experiment are quite dramatic. As soon as cellularization is complete and gastrulation should begin, the normally columnar epithelial cells of the cellular blastoderm round up, loosen their contacts with each other and form a multi-layered mesenchyme (Fig. 5). This disruption of the overall architecture of the embryo blocks the complex morphogenetic movements of gastrulation. At times one sees vestiges of normal gastrulation, such as partial apical constriction of the ventral furrow or partial germband elongation, but these are seen only infrequently

and never proceed to completion. This disruption is probably due in part to the loss of anchorage and polarity of the actin cytoskeleton seen in mutant embryos (Fig. 6), confirming that these are properties conferred by the adherens junction.

One surprising feature of the development of *arm*<sup>XP33</sup> mutants is that their cells later reassemble rudimentary epithelial and other multicellular structures (Figs. 5, 6, and 8). We suspect this is due to the residual Armadillo function provided by *arm*<sup>XP33</sup> mutant protein, rather than being the result of an Armadillo-independent process. *arm*<sup>XP33</sup>, while strong, is not null (Peifer and Wieschaus, 1990; these results). When zygotic expression of Armadillo reaches its maximum level at the extended germ band stage (Riggleman et al., 1989, 1990), the mutant protein made appears to allow some cells to reassemble epithelia, with adherens-like junctions at their apical ends. We believe that formation of epithelial balls results from residual function rather than Armadillo-independent assembly of junctions because mutant Armadillo protein accumulates at the site of these junctions (Fig. 8). Relatively low levels of Armadillo activity suffice for partial junction function, consistent with previous observations concerning the zygotic phenotype of the known *arm* alleles. We found it interesting that both epithelial balls and spindle-shaped cells arose at characteristic positions along the anterior-posterior axis. Cells must retain some information about their intended cell fates despite the loss of epithelial integrity and the disruptions caused by blocking gastrulation. Some cells go on to form vestiges of normal larval structures, such as the cuticular pieces secreted by epithelial balls. *arm*<sup>XP33</sup> mutant embryos also twitch in a fashion similar to the twitching caused by muscular contraction in wild-type embryos, suggesting that some differentiation of muscle and nerves may also occur.

### **Armadillo Is Required for Adherens Junction Assembly**

Biochemical evidence in vertebrates (Aberle et al., 1994; Hülsken et al., 1994; Jou et al., 1995) and *Drosophila* (Pai, L.-M., C. Kirkpatrick, and M. Peifer, unpublished data) suggests that Armadillo links  $\alpha$ -catenin and E-cadherin. We found that removal of Armadillo disrupts adherens junction assembly in embryos, dramatically altering the intracellular localization of both DE-cadherin and  $\alpha$ -catenin at the onset of gastrulation (Fig. 8). In the absence of Armadillo,  $\alpha$ -catenin is not found in adherens junctions but instead accumulates inside the cell, with little or no enrichment at the membrane. The normal enrichment of DE-cadherin in adherens junctions is lost. Levels of DE-cadherin inside cells are greatly increased, perhaps reflecting accumulation in endocytic vesicles. Reduction in Ca<sup>2+</sup> levels in cultured cells causes a similar redistribution of cadherins (Kartenbeck et al., 1991).

We suspect that in the absence of Armadillo,  $\alpha$ -catenin is no longer bound to DE-cadherin and is thus free in the cytoplasm. This disrupts anchoring of the actin cytoskeleton at the adherens junction. DE-cadherin, stripped of its two high affinity partners, is rapidly internalized. Steady-state levels of both  $\alpha$ -catenin and DE-cadherin remain near normal (Fig. 7), however and they rapidly reassemble into apparent adherens junctions when zygotic levels of

*arm*<sup>XP33</sup> mutant protein become sufficient to allow junction formation (Fig. 8). Relative levels of labeling suggest that Armadillo may act in substoichiometric ratios to stimulate formation of junctions, despite the fact that in vertebrate cells, the predominant subunit of the adherens junction is a 1:1:1 complex of E-cadherin,  $\beta$ -catenin, and  $\alpha$ -catenin (Ozawa and Kemler, 1992).

### **Armadillo and Adherens Junctions Maintain Cell Polarity Early in Development**

One feature of epithelia is that the cells are polarized, with defined apical and basolateral surfaces; lateral surfaces are further polarized, such that specific junctional types form in particular positions. Adherens junctions are thought to help initiate and maintain this polarity (McNeill et al., 1990). Depletion of functional Armadillo from embryos disrupted cell polarity, as seen by disruptions in the polarized accumulation of phosphotyrosine (Fig. 8), normally enriched in adherens junctions, and f-actin (Fig. 6), which normally accumulates most heavily in adherens junctions and at the basal surface. Both markers are polarized in their localization in mutant embryos until the completion of cellularization, suggesting that this initial polarization does not require adherens junctions. Instead, it may reflect differences in the nature of the original egg plasma membrane, which remains apical, and the newly created lateral plasma membrane. The localization of phosphotyrosine and f-actin is rapidly and dramatically altered in mutants once gastrulation begins. Unlike DE-cadherin, these markers remain greatly enriched at the plasma membrane, but become uniformly distributed around the circumference of the cells. The cells also lose their characteristically asymmetric columnar shape and become rounded in appearance.

During later stages in development, when a subset of the mutant cells reassemble rudimentary epithelial balls, both polarity markers, actin and phosphotyrosine, become specifically re-polarized in this subset of cells (Figs. 6 and 8). There is a strong correlation between reassembly of adherens junctions, as assayed by immunofluorescence to detect junctional components, and reacquisition of polarity, as assayed by distribution of phosphotyrosine and actin. The identity of the adherens junction-enriched protein to which phosphotyrosine is attached is unknown. These data suggest that it is not one of the known junctional components, as phosphotyrosine remains associated with the plasma membrane in the absence or near absence of Armadillo, DE-cadherin, or  $\alpha$ -catenin.

### **Adherens Junctions, the Zonula Adherens and the Development of Epithelial Cells**

We now know a great deal about the identity of the core components of the adherens junction, the nature of their interactions with each other, and the nature of their contributions to junction function in cultured cells and intact animals. Many questions remain. The adherens junction is both dynamic and complex. Morphological analysis in *Drosophila* demonstrates that the fully formed zonula adherens develops only late in embryogenesis (Tepass and Hartenstein, 1994); for several hours after the onset of gastrulation, cells are joined only by spot adherens junctions. It is

not until spot adherens junctions coalesce into the zonula adherens that the fully polarized state of the epithelial sheet is obtained. It is only at that point, for example, that septate junctions (probable equivalents of vertebrate tight junctions) are assembled (Tepass and Hartenstein, 1994). From the data presented here, it is clear that Armadillo is required for assembly of spot adherens junctions, and that these are required for cell adhesion and for polarity at early stages. The products of the *bazooka* and *stardust* genes also are essential for initial assembly of adherens junctions (Müller and Wieschaus, 1996)—the nature of the products encoded by these genes is thus of great interest. However, it is less clear how the zonula adherens is assembled and how this prompts the assembly of other junctional complexes, such as the septate junction. Certain gene products, such as Crumbs, clearly are required for cell polarity during later stages of embryogenesis (Tepass et al., 1990; Tepass and Knust, 1993; Wodarz et al., 1993), during and after assembly of the zonula adherens. However, Crumbs does not appear to be required for initial assembly of spot adherens junctions or for early cell polarity (Grawe et al., 1996; Tepass, 1996). Thus, the development of mature epithelial tissues is likely to require several steps, with later cells having more elaborate machinery than their ancestors.

We are particularly grateful to Drs. M. Takeichi and T. Uemura for generously sharing antibodies, fly stocks and unpublished data, and for helpful discussions. We are also indebted to J. Adam for asking the questions that resulted in the initiation of this project and for help with early experiments. We would like to thank J. Shields for keeping our lab operating and S. Whitfield for her superb photographic help. We would also like to thank Drs. E. Wieschaus, A. Müller, U. Tepass, and V. Hartenstein for sharing unpublished data, Dr. U. Tepass for providing fly stocks, and Drs. J. Carson and T. Perdue for help with scanning EM. Finally, we would like to thank E. Cox, M. Kinch, D. Kirkpatrick, G. Maroni, M. Turmidge, and one of the reviewers for insightful comments on the manuscript, and members of the Peifer lab for valuable suggestions and discussions.

R. Cox was supported by National Institutes of Health (NIH) Training grant 5T32 GM07092, and the project was supported by NIH grant GM47857 and by a grant from the Searle Scholars Program to M. Peifer.

Received for publication 16 February 1996 and in revised form 9 April 1996.

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