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The *Drosophila* genes *crumbs* and *stardust* are involved in the biogenesis of adherens junctions

Ferdi Grawe[1], Andreas Wodarz[2], Bill Lee[3], Elisabeth Knust[1] and Helen Skaer[3,*]

[1]Institut für Entwicklungsbiologie, Universität zu Köln, 50923 Köln, Germany
[2]Howard Hughes Medical Institute and Department of Developmental Biology, Beckman Center, Stanford University, Stanford, CA 94305-5428, USA
[3]Department of Zoology, University of Cambridge, UK

*Present address: University of Oxford, Department of Anatomy, South Parks Road, Oxford OX1 3QX, UK

SUMMARY

Morphogenetic movements of epithelia during development underlie the normal elaboration of the final body plan. The tissue integrity critical for these movements is conferred by anchorage of the cytoskeleton to the membrane at sites of cell adhesion, mediated by adherens junctions, initially spot and later belt-like, zonular structures, which encircle the apical side of the cell. Loss-of-function mutations in the *Drosophila* genes *crumbs* and *stardust* lead to the loss of cell polarity in most ectodermally derived epithelia, followed in some, such as the epidermis, by extensive apoptosis. Here we show that both mutants fail to establish proper zonulae adherentes in the epidermis. Our results suggest that the two genes are involved in different aspects of this process. Further, they are compatible with the hypothesis that *crumbs* delimits the apical border, where the zonula adherens usually forms and where Crumbs protein is normally most abundant. In contrast, *stardust* seems to be required at an earlier stage for the assembly of the spot adherens junctions. In both mutants, the defects observed at the ultrastructural level are preceded by a misdistribution of Armadillo and DE-cadherin, the homologues of β-catenin and E-cadherin, respectively, which are two constituents of the vertebrate adherens junctions. Strikingly, expansion of the apical membrane domain in epidermal cells by overexpression of *crumbs* also abolishes the formation of adherens junctions and results in the disruption of tissue integrity, but without loss of membrane polarity. This result supports the view that membrane polarity is independent of the formation of adherens junctions in epidermal cells.

Key words: *Drosophila*, *crumbs*, *stardust* cell polarity, epithelia, adherens junctions

INTRODUCTION

Epithelia are characterized by a pronounced apicobasal polarity and a highly integrated tissue structure. They separate different body compartments and control the vectorial exchange of molecules between them (see Rodriguez-Boulan and Nelson, 1989, for review). During development, the remodelling of epithelial tissues plays a pivotal role in the organisation of the final body plan. These functions of epithelia depend on a tightly integrated tissue structure, which is based on the presence of intercellular junctions. Some of these junctions, e.g. tight junctions and septate junctions, are unique to epithelia, while others, such as gap junctions, are common to other tissues as well. Adhesive junctions are more elaborated in epithelia than elsewhere, and include both cell-cell and cell-substrate junctions (see Garrod and Collins, 1992, for review). As well as their adhesive properties, these junctions serve as anchorage points for the cytoskeleton; actin filaments associate with adherens junctions and intermediate filaments with desmosomes. Filament attachment is mediated by an assemblage of specific proteins on the cytoplasmic face of the junction, called the cytoplasmic plaque. Attachment of cytoskeletal elements to the cytoplasmic plaque establishes an intercellular link by which forces can be transmitted between cells via the junctions. In this way, cell adhesion is coupled to the modulation of cell shape. In addition, recent evidence points to adherens junctions as sites at which signals can be transferred in both directions (reviewed by Rantsch, 1994; Peifer, 1995).

Analysis of the developing mouse embryo and experiments with cultured cells both suggest the importance of cell-cell adhesion for the establishment and maintenance of epithelia. In the mouse embryo, loss of a functional E-cadherin gene prevents the formation of the trophectoderm during mouse embryogenesis (Larue et al., 1994; Riethmacher et al., 1995). Conversely, transfection of DNA encoding uvomorulin (i.e. E-cadherin) into non-polarised cells induces the polarised distribution of other molecules, such as fodrin or the Na^+, K^+-ATPase to the sites of cell contacts (McNeill et al., 1990), a process that is probably mediated by the recruitment of both membrane and cytoplasmic proteins as well as cytoskeletal elements. These data suggest a close relationship between the

development of adherens junctions and mechanisms controlling epithelial cell polarity and tissue integrity. As the establishment and maintenance of epithelial tissues underpins morphogenesis, a deeper insight into the processes that govern the assembly of adherens junctions is necessary for our understanding of the molecular and cellular regulation of tissue development.

Adherens junctions (AJs) are multiprotein complexes, composed of transmembrane proteins (cadherins) and a variety of cytoplasmic proteins, including β-catenin, which is directly associated with cadherins. Other cytoplasmic proteins link the cadherin/β-catenin complex to actin filaments, e.g. α-catenin, vinculin, α-actinin or radixin (Huelsken et al., 1994; see Garrod and Collins, 1992; Gumbiner, 1993; Hitt and Luna, 1994; Luna and Hitt, 1992; Kemler, 1993 and Rantsch, 1994, for reviews). Despite their importance for maintaining the integrity of epithelial tissues, information about the various components of these junctions is limited and very little is known about the steps required for their assembly or the localisation signals that site them in appropriate regions of the membrane. Since many of the components associated with the junctions are already present, but distributed uniformly in cells prior to polarisation and junction formation, it has been suggested that their assembly could be triggered by extracellular and/or intracellular signals. For example, a change in the intra- or extracellular Ca^{2+}-concentration has been shown to trigger conformational changes in Ca^{2+}-dependent adhesion molecules, such as the desmosomal cadherins, thereby increasing their adhesive properties. In addition, the assembly of proteins might be regulated by differential phosphorylation, mediated by specific protein kinases or phosphatases (see Garrod and Collins, 1992, for review). There seems to be a close relationship between the state of protein phosphorylation and adhesion: overexpression of an oncogenic kinase, such as $pp60^{v\text{-}src}$, or treatment with inhibitors of tyrosine-specific phosphatases reduce adhesion and ultimately lead to the breakdown of AJs, which is likely to be correlated with phosphorylation of β-catenin (Matsuyoshi et al., 1992; Volberg et al., 1992; Behrens et al., 1993).

The *Drosophila* embryo provides an ideal system in which to dissect the sequence of events leading to the assembly of adherens junctions. The ultrastructure of junction formation during embryonic development has been described in great detail (Eichenberger-Glinz, 1979; Tepaß and Hartenstein, 1994a). Furthermore, a combined genetic and molecular approach has not only demonstrated that several of the proteins known to be involved in this process are conserved between vertebrates and *Drosophila*, e.g. E-cadherin, α- and β-catenin (Oda et al., 1993, 1994; Peifer and Wieschaus, 1990), but also uncovered novel genes and their products (reviewed in Knust, 1994). Two of these are the genes *crumbs* (*crb*) and *stardust* (*sdt*). Mutations in either of these genes lead to loss of cell polarity in most ectodermally derived epithelia, followed by breakdown of epithelial structure and extensive cell death. Genetic analysis has shown that both genes act in a common genetic pathway (Tepaß and Knust, 1990, 1993). The Crumbs protein is an integral membrane protein, which contains 30 EGF-like and four laminin A G domain-like repeats in its extracellular region, and is expressed on the apical side of all epithelia derived from the ectoderm (Tepaß et al., 1990). The small cytoplasmic portion of 37 amino acids is of crucial importance for the function of the Crumbs protein: truncation of this domain leads to a complete loss of function (Wodarz et al., 1993). Conversely, overexpression of just this domain has the same effect as overexpression of the whole protein, resulting in expansion of apical membrane at the expense of the basolateral membrane and extensive reorganisation of the cytoskeleton in epithelial cells (Wodarz et al., 1995).

In this paper, we present data showing that the loss of cell polarity in the epidermal primordium of *crb* and *sdt* mutant embryos is associated with a failure to establish the zonulae adherentes. In addition, we demonstrate that these junctions fail to develop when an altered polarity is induced by the overexpression of Crumbs. Our results further suggest different requirements for *crb* and *sdt* during the formation of the zonula adherens.

MATERIALS AND METHODS

Fly strains, generation of gynandromorphs and ectopic expression by means of the GAL4 system

The following fly strains were used: crb^{11A22}, crb^{8F105}, sdt^{7D22}, sdt^{EH} and Oregon R as wild-type. Mutant strains were kept over balancer chromosomes that were marked with a *ftz-lacZ* reporter gene in order to distinguish the homozygous mutant embryos.

For ectopic expression of *crb*, we made use of the GAL4 system described by Brand and Perrimon (1993). The following activator and effector strains were used: $GAL4^{559.1}$, which expresses GAL4 under the control of the *patched* promoter (Hinz et al., 1994); $GAL4^{daG32}$, which gives a more or less uniform expression pattern (Wodarz et al., 1995); UAS-crb^{wt2e}, which encodes the full-length Crumbs protein, and UAS-$crb^{intra\text{-}myc4a}$, which encodes a protein consisting of the membrane bound cytoplasmic domain of Crumbs (Wodarz et al., 1995). Unless otherwise stated, we crossed females from homozygous effector lines to males of homozygous activator strains to avoid undesired activation of the effector constructs by maternally provided GAL4 protein.

sdt gynandromorphs were produced by crossing w sdt^{EH}/*FM7, P[*w^+ *ftz-lacZ]* females to *R(1)2, In(1)*w^{vC}, w^{vC} *P{*ry^+*, ftz-lacZ} ct / In(1)dl-49, y l(1) w /* y^+ *Y* males (kindly provided by W. Janning). Since the ring-X chromosome is labelled with a *ftz-lacZ* reporter gene, gynandromorphs can easily be detected after *lacZ* staining by the differential appearance of the *ftz* stripes. Appropriate gynandromorphs were selected for sectioning. For details concerning the production and use of gynandromorphs the reader is referred to Janning (1978) and Ashburner (1990).

Transmission electron microscopy and confocal laser scanning microscopy

Transmission electron microscopy was essentially performed as described in Tepaß and Hartenstein (1994a), using a Zeiss EM 900 or a Philips 300 electron microscope. Confocal laser scanning microscopy was done according to Wodarz et al. (1995). Dilutions of primary antibodies were as follows: mouse anti-Armadillo 1:4 (kindly provided by M. Peifer); rat anti-DE-Cadherin 1:20 (kindly provided by T. Uemura). FITC-conjugated goat anti-mouse and goat anti-rat secondary antibodies (Sigma) were used at a dilution of 1:100. Embryos were mounted in Vectashield (Vector) mounting medium and viewed on a BioRad MRC 1000 confocal laser scanning microscope equipped with COMOS software (BioRad). Image processing and mounting of figure panels was done in Photoshop (Adobe) on Macintosh equipment. Images were printed on a Kodak XLS 8300 colour laser printer.

RESULTS

Embryos mutant for *crumbs* (*crb*) or *stardust* (*sdt*) show a common phenotype, in which epithelial cells of ectodermal origin and, in particular, those of the epidermis, lose their apicobasal polarity, resulting in the loss of epithelial integrity and cell death (Tepaß and Knust, 1990, 1993). However, it is evident that changes in cell polarity, shown by the mislocalisation of proteins that are targeted apically in wild-type embryos, occur some considerable time before clear morphological changes in cell structure can be seen by light microscopy (Wodarz et al., 1993). We therefore examined embryos by electron microscopy from stage 9 onwards in order to assess differences in the ultrastructure of ectodermally derived epithelia in wild type and in embryos mutant for *crb* and *sdt*. We found no gross changes in cell shape or the distribution of cell organelles in mutant embryos before the onset of apoptosis during stage 10 (Fig. 1A-C) and so we concentrated our attention on the development of intercellular junctions, on which the development and maintenance of epithelial integrity is known to depend (Fleming, 1992; Collins and Fleming, 1995).

Early development of junctions in the ectoderm of wild-type and mutant embryos

Ultrastructural analysis reveals that cellularisation of the blastoderm during stage 5 is characterised by the development of spot adherens junctions (SAJ) and gap junctions (GJ) on the lateral borders of the cell membranes (Tepaß and Hartenstein, 1994a). Shortly after this, belt or zonula adherens junctions (ZA) start to develop at the apical end of the lateral borders. SAJs and ZA have a similar appearance in thin sections but two features establish the belt-like nature of ZA. Firstly, both sides of a single cell always display an adherens junction in a single section (as in Fig. 1D) and, secondly, every section in a series reveals the presence of an adherens junction in the apical region of the lateral border. In contrast, SAJs may be present only on one border of a sectioned cell (as in Fig. 1E) and, in a series, they disappear after a few sections.

In stage 9 embryos, ZA are found on every border in the amnioserosa (Fig. 1D), while in the anlage of the epidermis there are still SAJs in this region (Fig. 1D inset). During the following stages, the number of SAJ diminishes and by the end of stage 10 almost every intercellular border in the ectoderm

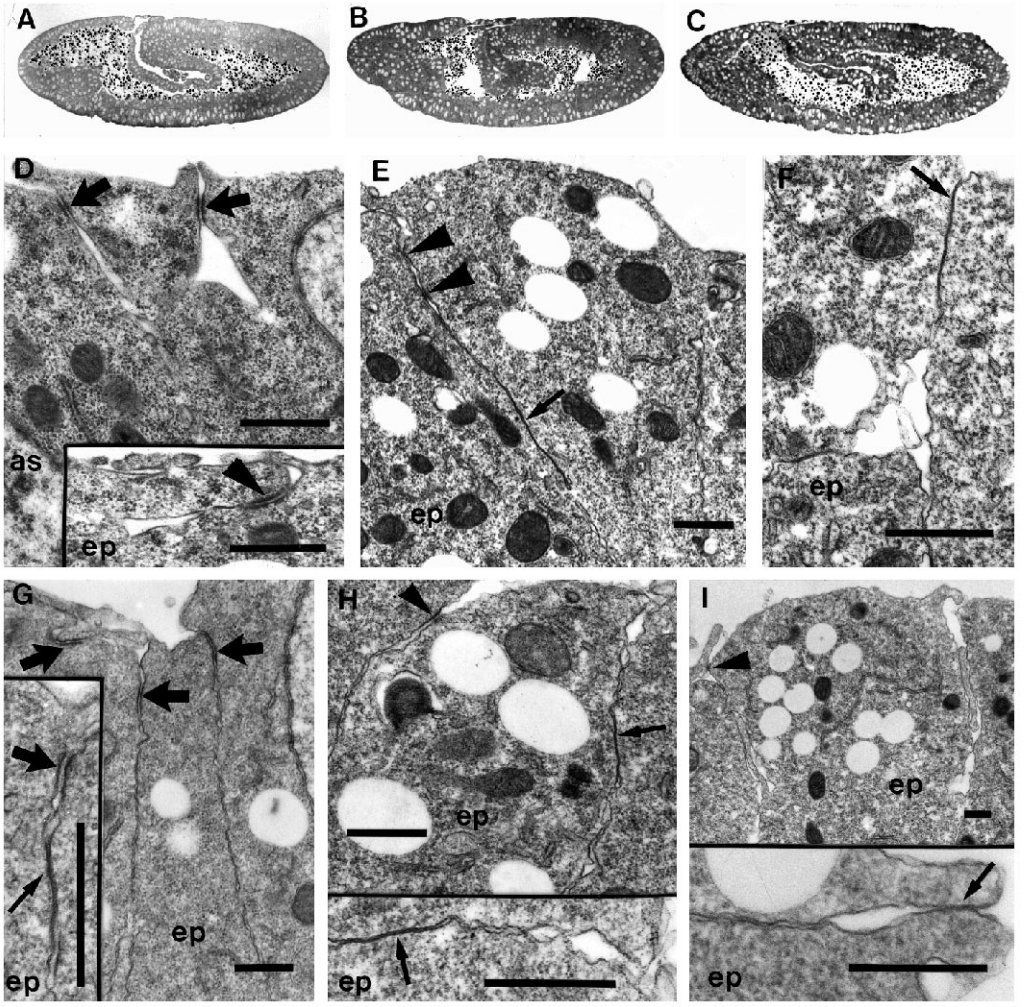

Fig. 1. Development of intercellular junctions in wild-type, *crb* and *sdt* embryos. (A-F) stage 9; (G-I) stage 10. (A,D,G) Wild type; (B,E,H) *crb*; (C,F,I) *sdt*. (A-C) Sagittal sections viewed by light microscopy reveal little alteration in epithelial organisation in the mutant embryos at this stage. Electron microscopy shows that ZA are already visible at the apical end of the amnioserosa cells in stage 9 wild-type embryos (D), while in the epidermis there are still SAJs in this region (D inset). ZA appear in the epidermis only during stage 10 (G), in a more apical position than GJs (G, inset). In embryos mutant for *crb*, the lateral borders of the epidermal cells appear normal at stage 9, with SAJ and more basal GJ (E), but both epidermal and amnioserosa cells of stage 10 lack ZA; there is either no junction at all at the apical end of the lateral border (H, shown at higher magnification in inset) or occasionally a weak SAJ can be seen (epidermis, arrowhead in H). Defects in the organisation of the junctions can be seen by stage 9 in embryos mutant for *sdt*. There are very few SAJ, so that GJ may extend to the apical end of the lateral border (F) and by stage 10 most borders lack AJ of any kind, although very small SAJ (I) and GJ (I inset) are occasionally seen as, amnioserosa; ep, epidermis; arrowhead, SAJ; thick arrow, ZA; thin arrow, GJ. The apical side of the epithelium is shown either to the top or to the right of each figure. The scale bar represents 0.5 μm. ZA and SAJ can be distinguished in the sections shown in this and the following figures by the presence of ZA on both sides of a single cell and of SAJ only on one side (cf Fig. 1D ZA with Fig. 1E SAJ).

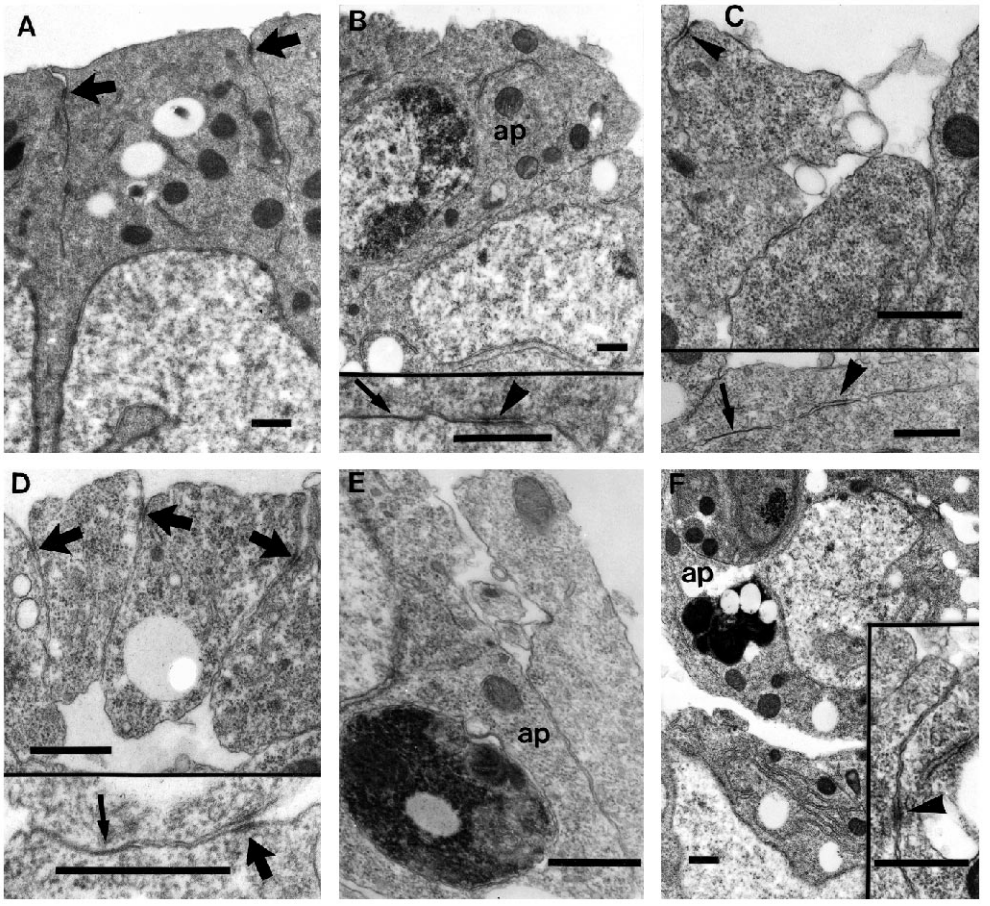

Fig. 2. Development of intercellular junctions in wild-type, *crb* and *sdt* embryos at stage 11. (A,D) Wild type; (B,E) *crb*; (C,F) *sdt*. (A-C) Epidermis; (D-F) amnioserosa. Electron microscopy shows an apical ZA on the lateral border of every cell of both the epidermis (A) and the amnioserosa (D) in wild-type embryos, with GJ situated more basally (D inset). ZA are not found in either the epidermis or amnioserosa of embryos mutant for *crb* or *sdt*. SAJ are occasionally found (C, arrowhead). These infrequent SAJ are sometimes found basal to the apical extremity of the lateral border with GJ lying basal to them (*crb*, B inset; *sdt*, C inset) and these SAJ are often very small (F inset). In other instances, borders lack AJ altogether, often when a cell on one side is dying (B,E,F). ap, apoptotic cell; arrowhead, SAJ; thick arrow, ZA; thin arrow, GJ. The apical side of the epithelium is shown either to the top or to the left (F) of each figure. The scale bar represents 0.5 μm.

is characterised by a single apically located ZA, with GJs found basal to them (Fig 1G and inset). The disappearance of SAJs and development of ZA is completed by stage 11 in the epidermis and by stage 10 in the amnioserosa (Fig. 2A,D) (Tepaß and Hartenstein, 1994a).

In embryos mutant for *crb* (identified as described in Materials and Methods), SAJs and GJs initially develop normally and the SAJs decrease in number during stages 9 and 10 just as they do in wild-type embryos. This is reflected in a generally normal appearance of the embryos at this stage in the light microscope (Fig. 1B). However, at the time that the ZA develop in wild-type embryos during stages 10 and early 11, in embryos mutant for *crb* these junctions fail to form at the apical end of the lateral borders (Fig. 1E,H) and, in some cases, SAJ persist in a more basal position (Fig. 2B inset). However, GJ are found in their normal position (Figs 1E,H; 2B inset). By late stage 11, mutant embryos present a very abnormal appearance in the light microscope, with dead cells erupting from the amnioserosa and epidermis (Tepaß and Knust, 1990, 1993). This corresponds to the presence of many apoptotic cells from stage 10 in the amnioserosa (Fig. 2E) and from late stage 11 in the epidermis (Fig. 2B).

In embryos mutant for *sdt*, the number of SAJ is always lower than in wild-type embryos so that as early as stage 9 they are very infrequent on the lateral cell borders (Fig. 1F). As in *crb* mutant embryos, the ZA fail to develop during stages 9-10 and only remnants of SAJ can be seen on the lateral borders of the epidermal primordium (Fig. 2C, inset) and amnioserosa cells during stage 11 (Fig. 2F, inset), when many apoptotic cells can be found in both these tissues (e.g. Fig. 2F). GJs develop and persist in *sdt* mutant embryos but are often localised closer to the apical end of the border than in wild-type embryos (Fig. 1F).

In order to compare the appearance of intercellular junctions between wild-type and *sdt* mutant cells within the same embryo, we examined the development of ZA in gynandromorph embryos in which some cells were wild type and others were hemizygous mutant for *sdt*. By labelling wild-type cells with a P-element carrying a *lacZ* marker located on the ring-X chromosome, it was possible to identify wild-type and mutant cells in whole embryos (see Materials and Methods), in semithin sections and, by extrapolation, in the electron microscope. Study of embryos at late stage 10 indicated that wild-type ectodermal cells have a small ZA at the apical extremity of every lateral border, frequently with a GJ immediately basal to it. In contrast, mutant cells show no ZA but occasional SAJs can be found. Many borders exhibited no adherens junctions at all (data not shown).

Later differentiation of ectodermal tissues

The structure of all ectodermally derived epithelia is affected to varying extents in embryos mutant for either *crb* or *sdt*. The majority of cells of the most severely affected tissues (such as the epidermis) die and those that survive fail to differentiate normally later in development. Other ectodermally derived epithelia, such as the tracheal system, salivary glands, parts of

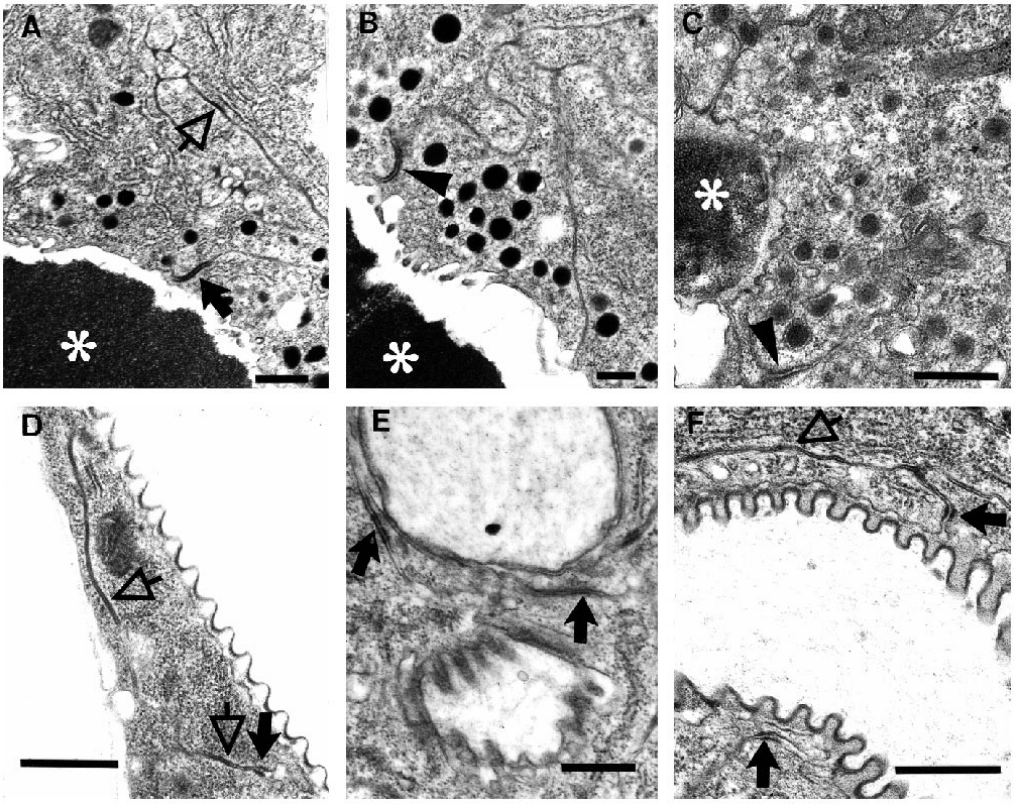

Fig. 3. Junctions in the salivary gland and tracheal epithelia of stage 16 embryos. Appearance of the salivary gland (A-C) and tracheal (D-F) epithelia in wild-type (A,D), *crb* (B,E) and *sdt* (C,F) embryos. In wild-type embryos, the lateral borders of both epithelia have an apical ZA below which GJ (not shown) and developing PSJ are found (A,D). Salivary gland cells in both wild-type and mutant embryos contain electron-dense secretory granules, which empty into the central lumen (*). Mutant salivary gland cells do not have ZA at the apical end of the border; there are occasional SAJ (arrowheads in B,C). The epithelium of the tracheal vesicles in mutant embryos appears well organised in that the cells show a normal apicobasal polarity with corrugated cuticle typical of this epithelium secreted apically**.** In both *crb* and *sdt* embryos AJ are present at the apical end of nearly every lateral border, suggesting that ZA may have developed. In addition PSJ are beginning to form basal to the AJ (open arrow in F). arrowhead, SAJ; large arrow, ZA; open arrow, developing PSJ. The scale bar represents 0.5 μm.

the foregut, hindgut and the Malpighian tubules, appear to be less affected in their overall structure (Tepaß and Knust, 1990, 1993). We examined trachea and salivary glands in stage 16 embryos to assess the development of intercellular junctions in these tissues.

In wild-type embryos, zonula adherens and gap junctions persist at the apical extremity of the lateral borders in ectodermally derived epithelial tissues, with the exception of the Malpighian tubules in which the ZA are lost during stage 17 and in early larval life (Skaer, 1993; Tepaß and Hartenstein, 1994a) (see Fig. 3 for salivary glands and trachea). Common to these tissues is the development of septate junctions, which appear, initially during stage 14, as irregular and infrequent electron-dense bars spanning the intercellular space of the lateral cell borders basal to the ZA and developing to form the mature structure soon after hatching (Tepaß and Hartenstein, 1994a). Developing septate junctions in wild-type tissues are illustrated in Fig. 3A,D.

In mutants for *crb* and *sdt*, the salivary glands are reduced in size (Tepaß and Knust, 1990, 1993) but the remaining cells form a polarised epithelium, with secretory vesicles released into the lumen as in the wild type (cf. Fig. 3A, wt, and Fig. 3B,C, *crb* and *sdt*). However, adherens junctions are not present on every lateral border (Fig. 3B *crb*, Fig. 3C *sdt*) and serial sections reveal that these infrequent adherens junctions are SAJ and not zonular (data not shown). In both mutants, the tracheae disintegrate and form small epithelial vesicles (Tepaß and Knust, 1990, 1993). In contrast to cells of the salivary gland, the remaining tracheal epithelium develops adherens junctions on the apical side of nearly every lateral border, suggesting that in this tissue ZAs have developed in most cases (Fig. 3E,F).

Development of junctions in embryos overexpressing Crumbs

Overexpression of Crumbs by means of the GAL4 system results in an expansion of the apical membrane domain accompanied by a reduction of the basolateral membrane domain, suggesting that *crb* is crucial in defining the apical surface of epithelial cells. This phenotype is induced by expression of either the full-length protein or a protein consisting of just the membrane-bound cytoplasmic domain (Wodarz et al., 1995). Although epithelial cells overexpressing Crumbs still exhibit cell surface polarity, the disruption of tissue integrity is as severe as in homozygous mutant *crb* embryos. We were therefore interested to analyse whether this phenotype is also associated with a failure to establish ZA. Epithelial cells in the ectoderm of stage 9 embryos, in which Crumbs is activated by a ubiquitously expressed GAL4 line ($GAL4^{daG32}$; Wodarz et al., 1995), show a reduction in the number of spot AJ in comparison to wild-type embryos of the same stage. In the amnioserosa, the number of AJs is also reduced; on some lateral borders, only very rudimentary AJs are found (cf. Fig. 4A with E (wild type)). Serial sections indicated that these AJs are SAJs and not zonular. At the end of germ band extension (from stage 11 onwards), most cell borders in the ectoderm do not contain any AJ (Fig. 4B), so that occasionally gap junctions reach the most apical regions. The few remaining AJs never form a proper ZA, but remain distributed along the lateral membranes, in some

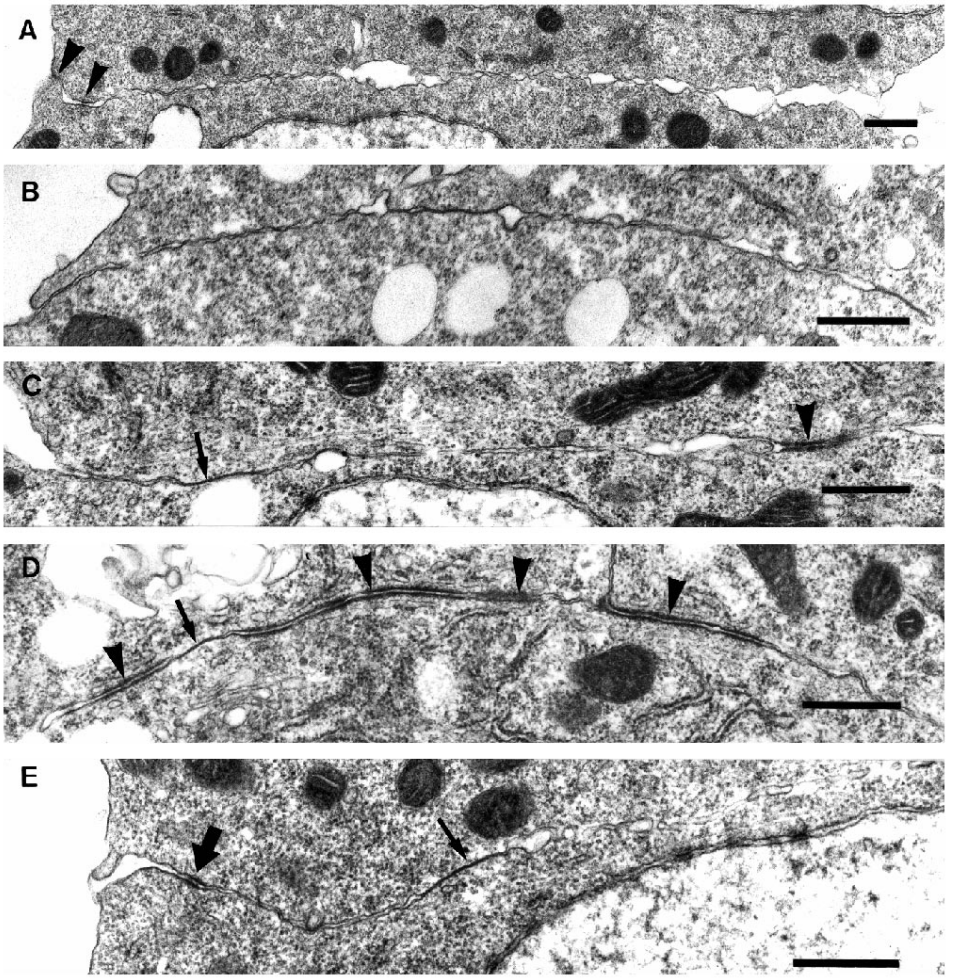

Fig. 4. Intercellular junctions in the amnioserosa and epidermis of embryos in which Crumbs is overexpressed. Genotype, UAS-crb$^{intra\text{-}myc4a}$/+; GAL4^{daG32}/+. (A) Stage 9, amnioserosa; (B-E) stage 11, epidermis. (A-D) Overexpression of *crb*; (E) wild type. (A) The amnioserosa lacks apical ZA (cf. Fig. 1D). In this case, there are two small SAJ in the apical region. (B-D). By stage 11, AJs are normally absent from amnioserosa and epidermal cell borders (B), though occasionally more basally located SAJ can be found (C,D). Arrowhead, SAJ; thick arrow, ZA; thin arrow, GJ. Apical is to the left. The scale bar represents 0.5 μm.

cases lying basal to the GJ (Fig. 4C,D). In addition, the membranes between adjacent cells tend to separate, so that instead of being aligned in parallel, they form bulges and protrusions (Fig. 4B). In contrast to *crb* mutant embryos, there is no increased incidence of cell death. Occasionally individual cells in the epidermis are pushed to the outside, indicating that the integrity and the cohesiveness of the tissue has been disrupted. In the amnioserosa, only occasional AJs and GJs are encountered. By stage 16, most epidermal cells lack AJ on their lateral borders (Wodarz et al., 1995).

Expression of DE-Cadherin and Armadillo in *crb* and *sdt* mutants and in embryos overexpressing Crumbs

The *Drosophila* homologues of β-catenin and E-cadherin, two components of the vertebrate adherens junction, have been identified and shown to be highly conserved when compared with their vertebrate counterparts (Peifer and Wieschaus, 1990; Oda et al., 1994). Armadillo and DE-Cadherin are associated with the zonula adherens in the embryo, although some Armadillo protein is also found in the cytoplasm (Peifer et al., 1994). However, both proteins are already enriched in the most apical region of the lateral membrane in stage 5 embryos, long before the zonulae adherentes can be detected at the ultrastructural level (data not shown). From stage 7 onwards, both proteins are concentrated in the most apical region of the cells, clearly outlining the cell surfaces (Fig. 5A,D). This subcellular localisation is maintained until the end of embryogenesis. In *crb* and *sdt* mutant embryos at stage 8, the distribution of both proteins in the amnioserosa is clearly abnormal: the majority is localised in a punctated pattern in the cytoplasm and the outlining of the cells is only visible as a diffuse, interrupted line, if at all (Fig. 5B,C). In the epidermis, the mislocalisation of Armadillo and DE-Cadherin is less obvious at this stage but, by stage 11, the amount of both proteins detectable at the apicolateral membrane is much reduced, resulting in dispersed spots of staining (cf. Fig. 5D to E,F). In general, the defects in the distribution of DE-Cadherin and Armadillo seem to be more severe in *sdt* than in *crb* mutant embryos of similar age.

In embryos overexpressing Crumbs, abnormal expression of Armadillo and DE-Cadherin can be observed from stage 10 onwards. Although, in stage 13 embryos, DE-Cadherin in the epidermis still forms the honeycomb-like pattern of expression typical of wild-type embryos, the outlining of cells is interrupted at many places (Fig. 5G), a defect that progresses until stage 16 (Fig. 5H). Sagittal optical sections of wild-type embryos reveal that DE-Cadherin is highly enriched in the most apical region of the lateral membrane, at the site of the zonula adherens (Fig. 5I). On overexpression of Crumbs however, DE-Cadherin is no longer restricted to the apicalmost lateral site, but can be detected on the basolateral membrane. In the amnioserosa, the effects of Crumbs overex-

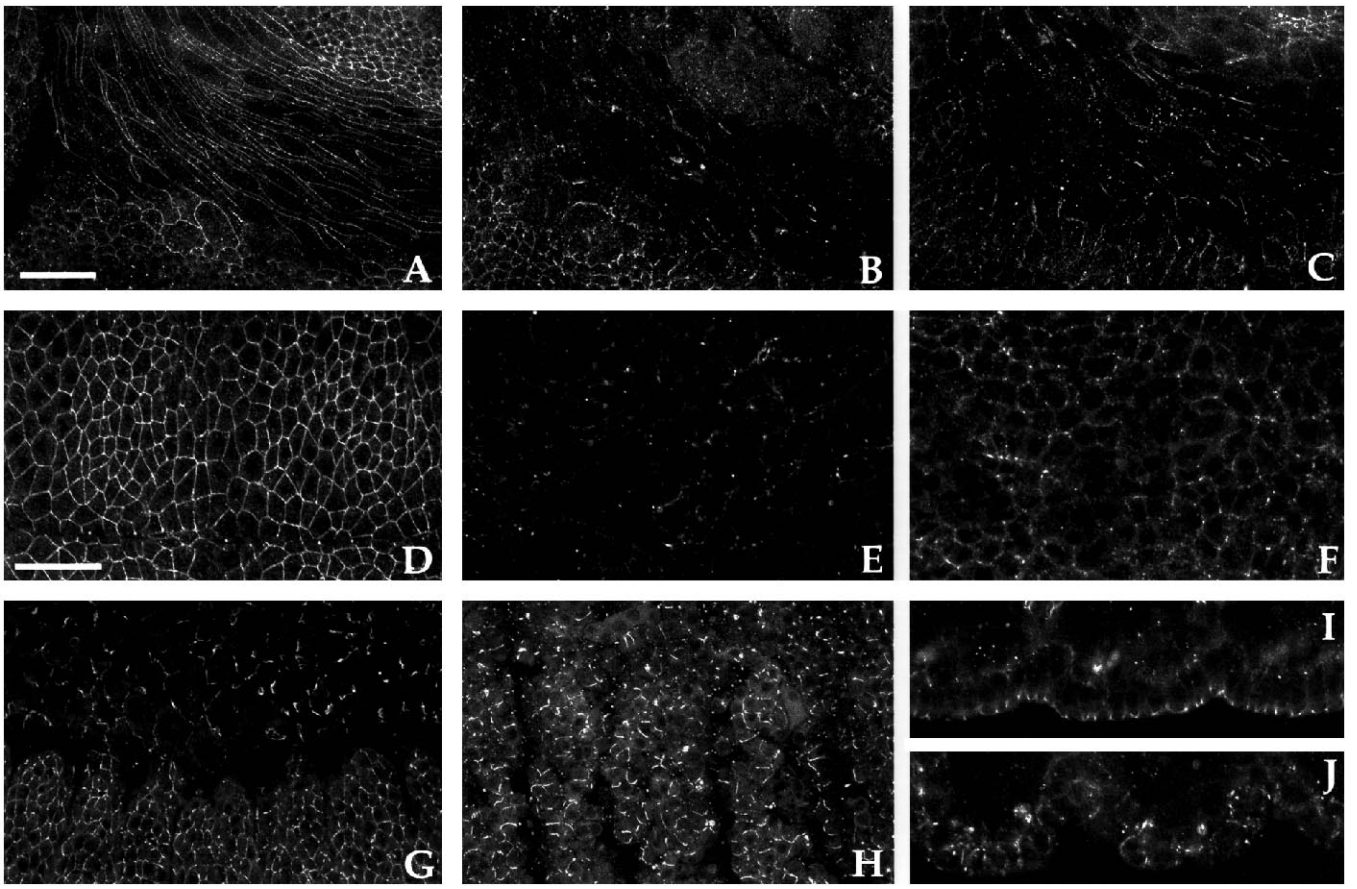

Fig. 5. Localisation of Armadillo and DE-Cadherin. (A,D,I) Wild-type embryos of stage 8, 12 and 15, respectively; (B,E) sdt^{EH}/Y of stage 8 and 12, respectively; (C,F) crb^{8F105}/crb^{8F105} of stage 8 and 12, respectively; (G,H,J) embryos overexpressing high levels of Crumbs (UAS-CRB^{wt2e}/+; $GAL4^{daG32}$/+) of stage 13 (G) and 15 (H,J). Embryos in A-C were stained for Armadillo, those in D-J for DE-Cadherin. (A) The amnioserosa of a wild-type embryo. The plane of focus is close to the apical surface of the cells. Armadillo is highly enriched at the apicolateral plasma membrane, thus outlining the cell shape. (B,C) In the amnioserosa of *sdt* (B) and *crb* (C) embryos, Armadillo staining is visible only as a spotlike pattern, indicative of the failure to form a zonula adherens in these cells. (D) The ventral epidermis of a wild-type embryo at stage 12, stained for DE-Cadherin. In the ventral epidermis, DE-Cadherin is concentrated at the zonula adherens, which forms a belt-like structure around each cell. (E,F) In *sdt* (E) and *crb* (F) embryos at the same stage (12), DE-Cadherin staining in the epidermis is reduced to scattered dots which are present all over the lateral plasma membrane. (G) Amnioserosa and dorsal epidermis of a stage 13 embryo overexpressing high levels of Crumbs. Note the much stronger disruption of the staining pattern of DE-Cadherin in the amnioserosa compared to the epidermis at this stage. (H) The epidermis of an older embryo (stage 15) overexpressing high levels of Crumbs. Compared to (G), the disruption of the zonula adherens has progressed further. (I, J) Sagittal optical sections of the epidermis of a wild-type embryo (I) and an embryo overexpressing Crumbs (both at stage 15). Note that DE-Cadherin is strongly enriched in the apicolateral portion of the plasma membrane of the wild-type embryo (I), while it is delocalised and present in ectopic positions in a Crumbs overexpressing embryo (J). Scale bar, 25 μm. The scale bar in A is valid for A-C, G and H and the scale bar in D is valid for D-F, I and J.

pression on DE-Cadherin can be observed earlier than in the epidermis, from stage 10 onwards. At stage 13, the staining outlining the cells is reduced to scattered fragments (Fig. 5G).

DISCUSSION

The data shown here complement and extend our knowledge concerning the temporal events that lead to the phenotypes characteristic of *crb* and *sdt* mutants. They also shed light on the relationship between cell polarity, junction formation and epithelial tissue integrity. The primary defect in *crb* and *sdt* mutants is the loss of cell polarity, demonstrated in *crb* embryos by the inability to localise apical proteins from stage 8 onwards (Wodarz et al., 1993). We show here that one consequence of this is the failure to establish a proper ZA. This is followed by defects, which have previously been described; namely that from the extended germ band onwards there is a loss of tissue integrity and extensive cell death in the epidermis (Tepaß and Knust 1990, 1993). While the lack of ZAs is likely to induce loss of tissue integrity, it cannot be the cause for cell death in the epidermal primordium of *crb* and *sdt* mutant embryos. Firstly, although embryos overexpressing Crumbs show a phenotype as severe as *sdt* mutant embryos in terms of ZA development and the consequent disorganisation of the epithelium, there is no increase in cell death (summarised in Fig. 6). Epithelial cells maintain their membrane polarity, even though the apical portion is expanded at the expense of the basolateral membrane domain (Wodarz et al., 1995). Secondly, in larvae mutant for the gene *fat*, the apical ZAs in the imaginal

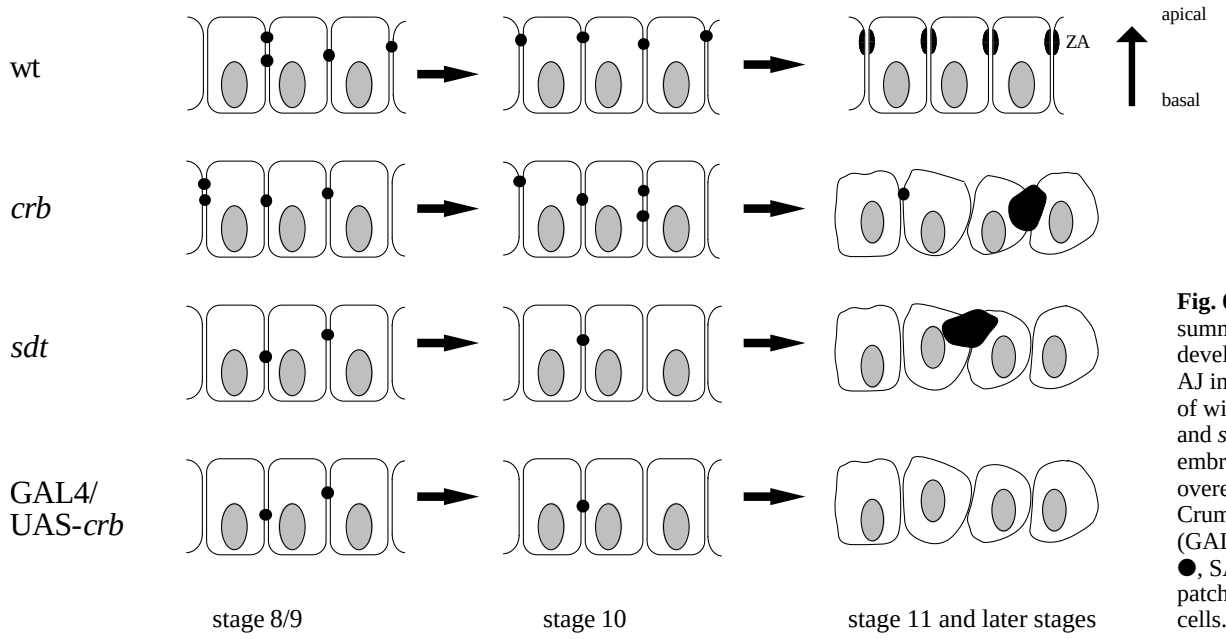

Fig. 6. Schematic summary of the development of the AJ in the epidermis of wild-type (wt), *crb* and *sdt* mutants and embryos overexpressing Crumbs (GAL4/UAS-CRB). ●, SAJ; ⦶, ZA; black patches, apoptotic cells.

discs are completely missing**.** Nevertheless the discs undergo extensive epithelial overgrowth and dead cells are only occasionally encountered (Bryant et al., 1988). Since the single layered epithelial structure is preserved in these overgrown discs and the cells secrete cuticle properly, their membrane polarity seems to be unaffected. These examples suggest that it is the loss of membrane polarity rather than the lack of ZAs that induces cells to die. In addition, the observation that membrane polarity, even a modified one, can be achieved in the absence of functional adherens junctions supports the view that the formation of the basolateral membrane domain, including the formation of junctions, and the establishment of the apicobasal axis are independent processes (see Eaton and Simons, 1995, for review). This has been demonstrated previously for individual *Xenopus* and mouse blastomeres (Müller and Hausen, 1995; Reeve and Ziomek, 1981).

So far, *Drosophila* genes known to affect the development of AJs and ZAs are rare. Besides *fat*, already mentioned above, two further candidates are *arm* and *DE-cadherin*, the products of which are the bona fide homologues of the vertebrate proteins, β-catenin and E-cadherin, respectively (Peifer and Wieschaus, 1990; Peifer et al., 1993; Oda et al., 1994). Both Armadillo and DE-Cadherin are concentrated at the sites of AJs in ectodermally derived epithelia. DE-Cadherin is encoded by the gene *shotgun.* The phenotype of zygotic mutations in this gene specifically affect processes that involve rearrangements of epithelia, such as the eversion and elongation of the Malpighian tubules**,** the outgrowth and branching of the tracheae (T. Uemura, personal communication), the outgrowth of the midgut epithelium (Tepaß and Hartenstein, 1994b) and the delamination of neuroblasts from the neuroectoderm. However, zygotic loss of *shotgun* function does not affect the ultrastructural appearance of the ZA (Tepass et al., 1996). Analysis of the consequences of the loss of Armadillo on the development of junctions is hampered by the strong maternal contribution of this gene product and the fact that *arm* is required for oogenesis (Wieschaus and Noell, 1986). However, defects observed in the ovaries of *arm* germ line clones (Peifer et al., 1993) as well as the presence of Armadillo in a multiprotein complex, resembling the vertebrate E-cadherin-catenin complex (Peifer, 1993; Oda et al., 1993), are consistent with Armadillo being a component of the AJs. Strikingly, in *crb* and *sdt* mutant embryos from stage 8 onward, only small amounts of both Armadillo and DE-Cadherin are associated with the plasma membrane as detected by immunolocalisation. We suggest that the early (stage 5) concentration of DE-Cadherin and Armadillo at the apicolateral plasma membrane is independent of Crumbs, since we could not detect any obvious abnormalities in *crb* and *sdt* mutant embryos at this stage. Furthermore, the Crumbs protein has not been detected at the plasma membrane before gastrulation (stage 6) and neither *crb* nor *sdt* have been shown to have an important maternal contribution (Tepaß and Knust, 1990, 1993).

According to the description of Tepaß and Hartenstein (1994a) and our own observations based on transmission electron microscopy presented here, the formation of the ZAs in the ectoderm of *Drosophila* is a gradual process. Spot AJs, which are initially scattered, concentrate in the apical region of the lateral membranes and finally form the subapical belt, the zonula adherens. The sequence of these events is compatible with the assumption that the SAJs coalesce to build up the ZA. In order to build a ZA at the correct site, a signal must be provided to mark and direct its assembly. We propose that *crb* may be responsible for the proper positioning of the ZAs in the subapical region. This suggestion is consistent with the localisation of the Crumbs protein in wild-type embryos, in which it is highly enriched in the apicolateral region of ectodermal cells from stage 6 onwards (Wodarz et al., 1995). In the absence of molecular data on proteins interacting with Crumbs, we can only speculate about the nature of this signal. Since the small cytoplasmic domain of Crumbs is essential for its function and overexpression of just this domain leads to the loss of the ZA, it appears to be the crucial player in this process. By analogy with other transmembrane proteins with short cytoplasmic tails devoid of domains with enzymatic activity, e.g. cadherins and integrins, we consider it most likely that the C-terminus of

Crumbs is connected to the underlying membrane cytoskeleton. Assuming that the cytoskeletal protein(s) that bind to Crumbs are distinct from the ones that bind to cadherins and integrins, the expression domain of Crumbs would thus delimit the range of the apical membrane cytoskeleton. Expanding this model further, it could be the interface between the apical and the lateral membrane cytoskeleton that serves as the positional cue to define the site of the ZA. The identification of proteins that interact with Crumbs will give us deeper insight into the molecular basis of this complex process, which is of crucial importance for epithelial morphogenesis in all higher organisms.

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