Zygotic *Drosophila* E-cadherin expression is required for processes of dynamic epithelial cell rearrangement in the *Drosophila* embryo

Tadashi Uemura,¹ Hiroki Oda,¹ Rachel Kraut,^{2,5} Shigeo Hayashi,³ Youhei Kataoka,¹ and Masatoshi Takeichi^{1,4}

¹Department of Biophysics, Faculty of Science, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-01, Japan; ²Institut für Entwicklungsbiologie, Universität zu Köln, D-50923, Köln, Germany; ³National Institute of Genetics, Mishima, Shizuoka-ken 411, Japan

Dynamic epithelial reorganization is essential for morphogenesis of various organs. In *Drosophila* embryos, for example, the Malpighian tubule is generated by cellular rearrangement of a preexisting epithelium and the tracheal network is formed by outgrowth, branching, and fusion of epithelial vesicles. Here we report that the previously identified locus *shotgun* (*shg*) encodes DE-cadherin, an epithelial cell–cell adhesion molecule of the classic cadherin type and that zygotic *shg* mutations rather specifically impair processes of the dynamic epithelial morphogenesis. In the mutants, the Malpighian tubule disintegrated into small spherical structures, and the tracheal network formation was blocked in selected steps. The malformation of these organs could be rescued by overexpression of DE-cadherin cDNA under a heat shock promoter. Unexpectedly, the zygotic null condition did not severely affect general epithelial organization; most epithelial tissues maintained not only their cell–cell associations but also their apicobasal polarity in the mutants. The zygotic null mutant retained a certain level of maternally derived DE-cadherin molecules until the end of embryogenesis. These results suggest that zygotic DE-cadherin expression is critical for the rearrangement processes of epithelial cells, whereas the maternally derived DE-cadherin may serve only for the maintenance of the static architecture of the epithelia.

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Epithelial cells can reposition themselves without breaking the cell layer. This type of cellular rearrangement plays an important role in the production of tissues or organs of diverse morphology (for review, see Gumbiner 1992). A well-known example is amphibian gastrulation, in which a process of intercellular movement, called convergent extension, causes a dramatic elongation of the mesodermal tissue (Keller et al. 1992). In *Drosophila* embryos, a similar mechanism operates for morphogenesis of tubular organs. These include Malpighian tubules (MTs) and probably tracheal trees as well, both of which are of ectodermal origin and simple epithelial monolayers. MTs arise from the posterior region of the hindgut (for review, see Skaer 1992, 1993). Primordia of the tubules first grow out by cell divisions, and when the proliferation is complete, 12–14 cells encircle the lumen. Subsequently the cells start shifting

their relative positions, generating elongated tracts with only two to three cells surrounding the lumen. Embryonic tracheal development is initiated by invagination of a cluster of cells in each of hemisegments T2 through A8 (for review, see Manning and Krasnow 1993). Each cluster gives rise to one metameric unit, which undergoes branching and outgrowth. Then a subset of branches fuses to one another to establish the basic framework. Once the invagination takes place, no further mitosis is observed, so this tracheal patterning most likely depends on cell rearrangement and/or cell shape change, although cellular mechanisms for individual branching processes have not been studied in detail.

In expectation of its possible functions in epithelial morphogenesis, we identified previously a *Drosophila* counterpart of the major vertebrate epithelial cadherin (E-cadherin) and designated it as DE-cadherin (Oda et al. 1994). The vertebrate E-cadherin belongs to a subgroup of the cadherin superfamily (for review, see Takeichi 1995). Members of this subfamily are named classic cadherins and they form multiprotein complexes with at

⁴Corresponding author.

⁵Present address: Institute of Molecular and Cell Biology, National University of Singapore, Singapore 0511, Malaysia.

least two classes of cytoplasmic molecules, α -catenin and β -catenin. This assembly is essential for the cell adhesion activities of cadherins (Hirano et al. 1992; Kawanishi et al. 1994; Oyama et al. 1995). Besides having structural similarities between itself and the vertebrate classic cadherins, *DE*-cadherin was shown to mediate cell adhesion in vitro and bind catenins, *D α* -catenin (Oda et al. 1993) and Armadillo (Arm), a β -catenin homolog (Peifer and Wieschaus 1990; McCrea et al. 1991). Arm was originally discovered as the product of a segment polarity gene belonging to the *wingless* (*wg*) class and is considered to have dual roles as an element of the adhesion apparatus and a transducer of the Wg signal reception (for review, see Hinck et al. 1994; Peifer 1995). *DE*-cadherin is expressed in all embryonic ectodermal epithelia, subsets of neural and mesodermal cells, and endodermal cells (Oda et al. 1994; H. Oda, and T. Uemura, unpubl.). At boundaries between epithelial cells, *DE*-cadherin is localized at the position of the actin-based zonula adherens (ZA); this suggests that *DE*-cadherin is a component of ZA, as is the case for the vertebrate E-cadherin (Tsukita et al. 1992; Tepass and Hartenstein 1994a). In invertebrates, three other molecules of the cadherin superfamily were reported: Fat (Mahoney et al. 1991), Dachshaus (Clark et al. 1995), and the toxin receptor BT-R₁ (Vadlamudi et al. 1995). However, their roles in cell adhesion remain to be investigated.

Vertebrate classic cadherins have been shown to be crucial for the integrity of polarized epithelial architecture (for review see Eaton and Simons 1995). This was demonstrated by a number of methods, including cDNA transfection of cell lines (McNeill et al. 1990; Watabe et al. 1994), expression of dominant-negative constructs (Kintner 1992; Levine et al. 1994; Hermiston and Gordon 1995; Lee and Gumbiner 1995), injection of antisense oligonucleotides (Heasman et al. 1994), and targeting of the mouse gene (Larue et al. 1994; Riethmacher et al. 1995). However, functions of cadherins in dynamic organogenesis have not been critically investigated in vivo by means of loss-of-function mutations. In this study we have isolated and characterized complete or partial loss-of-function mutants of the *DE*-cadherin gene. Phenotypes of these mutants indicated that zygotic expression is crucial for processes of cell rearrangement, including the extension of MTs and tracheal tree patterning. Unexpectedly, these epithelial tissues did not dissociate into single cells in the zygotic null mutant, nor were most of the other epithelial organs so severely affected by the mutation.

Results

DE-cadherin is encoded by *shotgun*

We cytologically located the *DE*-cadherin gene (*cadE*) very close to or in the chromosomal band 57B13-14. We found that two partially overlapping chromosomal deletions, *Df(2R)D17* (O'Donnell et al. 1989) and *Df(2R)E2* (Kraut and Campos-Ortega 1996), uncovered this gene (Fig. 1A). The transheterozygote of these two deficien-

Figure 1. (A) Cytological map of *cadE/shg* and deficiency chromosomes. *cadE/shg* is located within the 57B region on the second chromosome. Extents of deficiencies in individual lines are represented by solid lines. In a transheterozygote of *Df(2R)E2/Df(2R)D17* (*E2/D17*), the region of 57B5-6 to 13-14 is deleted. This drawing of polytene chromosome bands is modified from that in O'Donnell et al. (1989). (B) Genomic map of the *DE*-cadherin gene [(H) *Hind*III, (Hp) *Hpa*I]. In the *shg*^{P10469} chromosome, the PZ-element is inserted into the 5'-untranslated portion of the transcription unit (TU). RF1 and RF2 represent genomic regions of the plasmid rescue fragments. RF1 was rescued by *Xba*I (X) and *Spe*I (S) digestion, and RF2 was isolated by *Xba*I and *Nhe*I (N) digestion.

cies (*E2/D17*) thus represented a complete loss-of-function mutant for *cadE*, and it was designated as the zygotic null mutant or simply null mutant in this study. Within that overlap interval, we mapped an embryonic lethal mutation, *shotgun* (*shg*), which had been isolated by mutant searches for aberrant cuticle patterns (Nüsslein-Volhard et al. 1984). As described below, we isolated a P-element allele of *shg* in which the transposon was inserted in the *DE*-cadherin gene. Furthermore, we could rescue *shg* phenotypes by *DE*-cadherin cDNA expression. Therefore, *shg* most likely encodes *DE*-cadherin.

Besides the null mutant, we studied three embryonic lethal *shg* alleles (*shg*², *shg*^{E17B}, and *shg*^{SHN-1}), and one semilethal allele (*shg*^{P10469}, see Materials and methods). The embryonic lethal alleles were partial loss-of-function mutants; *shg*² showed intermediate defects and *shg*^{E17B} and *shg*^{SHN-1} gave weaker phenotypes. The *shg*^{P10469} had an insertion of a PZ element (Moldzik and Hiromi 1992) behind nucleotide 265 in the 5'-untranslated region of the *DE*-cadherin cDNA (Fig. 1B). Revertant analyses confirmed that this insertion did cause a

shg mutation: Of 32 independent revertant lines, 10 were completely viable both in a homozygous condition and in a *trans* relation to *shg*^{P10469}. Genomic Southern analyses indicated that the excision appeared to be precise in eight strains and near precise in 2 of the 10 strains (data not shown). The remaining 22 lines were homozygous lethal, and most of them produced embryos of weak *shg* phenotypes.

We immunostained mutant embryos for DE-cadherin. In ectodermal epithelia of the wild-type embryo, DE-cadherin was restricted to an apical position of cell-cell contacts, and its distribution was segregated from the territory of fasciclin III, a marker for the septate junction, which is located in the subapico-lateral region (Patel et al. 1987; Woods and Bryant 1993; see Fig. 2A). The *shg*^{E17B} (Fig. 2B), *shg*^{SHN-1}, and *shg*^{P10469} embryos exhibited normal apical distribution of DE-cadherin, but the signal intensity in the *shg*^{P10469} homozygotes was slightly reduced compared to that in heterozygotes. Embryos of *E2/D17* showed no intense immunostaining for DE-cadherin (Fig. 2C), although maternally derived molecules could be detected by Western analysis as described in the last section of this paper. In this null mutant, fasciclin III patterns were normal (Figs. 2E,F), suggesting little effect of this mutation on septate junction formation. *shg*² showed an abnormal staining pattern for DE-cadherin; most signals were diffusely scattered in the

cytoplasm although its apical concentration was also observed to some degree (Fig. 2D). Western analyses indicated that the *shg*² mutant cadherin molecule was partly degraded in vivo (Fig. 8C, below). The *shg*² product is probably mutated in such a way as to become more susceptible to proteolysis.

DE-cadherin is present in all epithelia in the normal embryo; therefore, extensive epithelial defects had been expected in the mutants. However, gross disorganization of epithelial tissues was limited to the head and ventral epidermis, Malpighian tubules, and tracheal ducts. In the null mutant embryo, ventral and head regions failed to form contiguous epidermis; its disruption became obvious at stage 11, and most cuticles were missing in those regions at the end of embryogenesis (Fig. 3B). In the remaining ventral cuticle, some denticles were formed and they were flanked by naked regions. This pattern was not similar to that of a strong loss-of-function *arm* mutant that produced ectopic denticles at the expense of naked regions (Klingensmith et al. 1989). The cuticles of the *shg*² mutant were similarly destroyed and tended to wrinkle. In 50% of the weak *shg*^{E17B} mutants, one or two small holes per embryo were seen in the cuticle (Fig. 3C). Mutant embryos of all the alleles showed defects in head involution and did not hatch, whereas no rupture was observed in their dorsal epidermis, as confirmed by staining for fasciclin III (Fig. 2F).

Figure 2. Subcellular localization of DE-cadherin and fasciclin III in the deficiency and *shg* mutant embryos (stage 15). (A) A wild-type hindgut was double-labeled for DE-cadherin (green) and a marker for the septate junction, fasciclin III (red). (B–D) DE-cadherin staining of hindguts in *shg*^{E17B} (B), *E2/D17* (C), and *shg*² (D). Confocal images were adjusted to increase brightness for C and D relative to B. (E,F) Staining for fasciclin III in the hindgut (E) and dorsal epidermis (F) of the deficiency embryo. Note apparently normal distribution of fasciclin III in these tissues. Arrows in F demarcate the dorsal midline. Scale bar, 30 μ m in A and 50 μ m in B–F.

Figure 3. Ventral cuticle patterns of the wild type (A), *E2/D17* (B), *shg*^{E17B} homozygote (C), and heat-shocked *E2/D17* carrying *hsp-cadE* transgene (D). The deficiency embryo lacks most of the head and ventral cuticles (B). Arrowheads in B point to denticles generated, and the arrow in C indicates a small hole. The *hsp-cadE* expression rescued the ventral cuticle phenotype, but head involution was still defective as shown by the exposed head skeleton (arrow). (B–D) Composite pictures of different focal planes of the same embryos. Anterior is to the top. Scale bar, 100 μ m.

To study whether the mutant phenotypes could be rescued by *DE*-cadherin expression, we attempted expression of *DE*-cadherin cDNA under a heat shock promoter (*hsp-cadE*). In both the null and *shg*² backgrounds, the cDNA expression restored the destroyed ventral cuticles, although the head defect was not recovered perfectly (Fig. 3D). In the following three sections, we focus on the phenotypes of MTs and tracheal ducts.

Disruption of MTs in *shg* mutants

The development of the MT is initiated by local evagination from the hindgut primordium (Skaer 1993). Following cell proliferation, cell rearrangement drives tubular elongation. Both mechanisms are regulated by specialized cells known as tip cells (Skaer 1989). As mentioned above, the MT structure was destroyed in the *shg* null mutant (Fig. 4B). However, the tip cells were present and differentiated into neurons in this mutant, as in the wild type (data not shown). We studied malformation of this organ by use of an antibody to a transmembrane protein, Crumbs (Crb), located at the outermost apical region of cell–cell boundaries as well as on the luminal surface (Tepass and Knust 1993; Wodarz et al. 1995). A structural abnormality of the MT was first detected at late stage 12 or early stage 13, when the proliferation is still continuing in the wild type. At these stages, instead of having the slender MT morphology of the normal embryo (Fig. 4C), the MT of the null mutant was rounded (Fig. 4D) and the single-layered epithelium was locally kinked (Fig. 4E). When the mutant entered the initial stage of cell rearrangement (late stage 13), the tissues started falling apart and eventually disintegrated into small cell clumps (Fig. 4G), although they did not disperse into single cells even at stage 17. Each spherical structure was composed of several cells, and cells in the aggregate adhered to each other. Interestingly, the individual aggregate was a closed vesicle with Crb concentrated on its inner surface. Therefore, the MT cells appeared to preserve apical polarity.

Further evidence for the conservation of cell polarity was provided by monitoring intracellular distribution

of a membrane cytoskeletal element, β_{Heavy} -spectrin (Thomas and Kiehart 1994). When embryos were fixed by heat methanol treatment (Miller et al. 1989), the cytoplasmic pool of β_{Heavy} -spectrin was washed away, resulting in an exclusive localization of the remaining molecules at an apical site of cell–cell contacts (Thomas and Kiehart 1994; as shown in Fig. 4H). In the mutant MT cell cysts, the β_{Heavy} -spectrin-enriched domain was located inside the aggregates, as seen in the case of Crb staining (Fig. 4I, arrows).

At the end of embryogenesis, the MT cells secrete uric acid, which accumulates in the lumen and thus makes this organ visible under polarized light. The null mutant produced no trace of the tubular structure (Fig. 5B); as opposed to the wild-type, elongated tracts filled with the crystal (Fig. 5A). A small fraction ($\sim 10\%$) of *shg*² embryos was able to generate short and discontinuous tubes (Fig. 5C). In the mutant embryos with the null or *shg*² genetic background, the induction of *hsp-cadE* rescued the organism from the MT malformation; an example of a rescued *shg*² is shown in Figure 5D.

Defective outgrowth and fusion of tracheal branches in *shg*

The *shg* mutation blocked specific steps of another tubulogenesis, that is, tracheal formation. To study fine structures of the tracheal network pattern, we used an antibody to an anonymous component that is deposited into the lumen (Klämbt et al. 1992). This staining allows detection of morphological changes of the trachea in each metamere unit, including its branching, outgrowth, and fusion (Fig. 6A,C,G). During development, the antibody first labels a subset of branches, the dorsal longitudinal trunks (DTs), just before their fusion at stage 13 (Fig. 6A). In the null mutant, DT cells started to migrate in the right direction, but the branch extensions were retarded (Fig. 6B). By the time DT fusion had already been completed in the wild-type embryo (Fig. 6C), 87% (91 of 105) of the mutant DTs still had not reached the more anterior metameres (Fig. 6D). Embryos with this “stall” phenotype could be rescued partially by the *DE*-

Figure 4. Disintegrated malpighian tubules (MTs) in the deficiency embryo. (A,B) Dorsal views of the wild-type (A) and the *E2/D17* (B) embryos at stage 15. Nuclei of MTs were visualized with an enhancer trap marker A154 (Bellen et al. 1989). In the mutant, the tract was converted into discontinuous cell aggregates (arrow). (HG) Hindgut. (C–G) Immunostaining of wild-type (C,F) and deficiency (D,E,G) embryos with anti-Crb antibody. (C,D) Dorsal views of stage 13 embryos. (E) A dorsolateral view of a deficiency embryo slightly older than that in D. (F,G) Dorsolateral views of late stage 15 embryos. In the mutant, cells of the MT formed spherical structures with a polarized Crb distribution (arrow). Elements of the dorsal longitudinal trunk (DT) of the tracheal system contacted each other, but they were not interconnected (arrowheads). (H,I) Distribution of β_{Heavy} -spectrin in the wild-type (H) and the deficiency embryos (I) fixed by the heat methanol method. Dorsal views of stage 15 embryos. β_{Heavy} -spectrin is located at the apical pole of cell–cell boundaries. In the deficiency embryo, β_{Heavy} -spectrin was concentrated on inner faces of cysts derived from disrupted MTs (arrows). The staining pattern in the hindgut was not altered. (A,B,F,G,H,I) Composite pictures of different focal planes. Anterior is to the left. Scale bar, 135 μm in A and B, 35 μm in C–G, and 50 μm in H and I.

isks). On the other hand, *shg* mutations did not essentially affect other aspects of tubular elongation. Every mutant metamere unit gave off shoots in the dorsoventral direction as well as visceral branches (Fig. 6C,D, arrow), although some of these tubes occasionally generated discontinuous points (Fig. 6F, asterisks).

Like developing MTs, the blind ends of outgrowing tracheal branches are associated with tip cells (Manning and Krasnow 1993). One class of the tip cells is termed homotip, and these are the leading cells of certain branches like DTs and LTs. We found that an antibody to the gene product of *escargot* (*esg*) stained nuclei of these homotip cells (Fuse et al. 1994) (Fig. 6I). Although roles of the tip cells in tracheal development have not been established, physical interactions between them precede the fusion (Manning and Krasnow 1993; S. Hayashi, unpubl.). In the *shg* null mutant, we could detect cells expressing *esg* as in the wild type (Fig. 6J), indicating that this mutation had no effect on tip cell differentiation.

Tracheal branching patterns in *arm* and *wg* mutants

As cadherin function requires β -catenin (Kawanishi et al. 1994; Oyama et al. 1995), we examined tracheal patterning phenotypes in mutants of *arm*, which encodes a *Drosophila* β -catenin (Peifer and Wieschaus 1990; McCrea et al. 1991). Tracheal phenotypes of *arm* were similar, though not identical, to those of *shg* (Figs. 7A–C). In one *arm* null mutant, *arm*^{YD35}, DTs were completely missing and LTs did not link to each other at all (Fig. 7A,B). In an *arm* hypomorph, *arm*^{H8.6}, DTs exhibited irregular width, and the connections between LTs were incomplete (Fig. 7C). We also attempted to determine whether the role of Arm in tracheal development is dependent on the signaling molecule Wg, as Arm is known to function downstream of Wg in the pattern formation of segment polarity (Noordermeer et al. 1994; Peifer et

cadherin cDNA expression (Fig. 6E). In *shg*² embryos, DTs fused partly, but the expansion of the tube diameter was arrested at every fusion point and the trunk remained constricted (Fig. 6F, arrows). This pattern was extreme in the weakest allele *shg*^{E17B}, in which all of the DT joints exhibited the bottleneck shape (Fig. 6H, arrows). Connections between not only DTs but also lateral longitudinal trunks (LTs; Fig. 6C, arrowhead) were hindered in these mutants. LT interconnection was finished before stage 16 in the wild type (Fig. 6G, arrowhead). However, even at stage 16, no LT fusion took place in the null mutant and 40% (38/96) of the LTs were not linked in the *shg*^{E17B} embryo (Fig. 6H, aster-

Figure 5. Structures of MTs in *shg* mutants and a phenotypic rescue by *DE-cadherin* cDNA expression. MTs at the end of embryogenesis or in first-instar larvae were observed under polarized light. (A) Wild type; (B) *E2/D17* did not display any tubular organization; (C) generation of short tracts in a *shg*² homozygote; (D) heat shock-treated *shg*² embryo that had the *hsp-cadE* transgene. The induction of *DE-cadherin* rescued both MTs and a tracheal network (arrowhead). The recovered tracheal branches at this stage are visible if filled with gas. In A, tracheal trees were out of focus. D is a composite panel of two different focal planes. All embryos are oriented with anterior to the left and dorsal down. Scale bar, 50 μ m.

al. 1994; Siegfried et al. 1994). In contrast to the indistinguishable ventral cuticle patterns in the *arm* and *wg* mutants, tracheal defects were quite different between these two mutant embryos. In a *wg* null mutant, *wg*^{CX4}, the entire dorsolateral ectoderm shows a transformation toward tracheal cells, but most of them do not invaginate and remain at the surface (Fig. 7D star; P. Green and V. Hartenstein, pers. comm.). DT-like branches were, however, formed partially on each side of the body (Fig. 7D, arrow), indicating that DT formation does not depend on *wg* activity completely. Therefore, the role of *Arm* in tracheal morphogenesis is at least partly separate from that of *Wg*. Although the tracheal system was defective in both *shg* and *arm* mutants, MTs extended normally without any breakage in *arm*^{YD35}.

Maternally derived *DE-cadherin* and *catenins* in the *shg* zygotic null mutant

In *shg* mutants, severe destruction was not seen in epithelial organs of the intestinal tract other than the MT. Invaginations of the salivary gland, foregut, and hindgut primordia proceeded normally, and their epithelial monolayers were not torn, at least at a gross level. The null mutants developed an esophagus and proventriculus from the foregut primordia as in the wild type, although their shape was slightly distorted. All of these ectodermal epithelia showed typical apical distributions of both *Crb* and β_{Heavy} -spectrin (for β_{Heavy} -spectrin, see Fig. 4I).

In an attempt to understand why severe phenotypes were limited to particular organs, we studied whether the zygotic null mutant produced *DE-cadherin* proteins

from maternal transcripts. Unhatched embryos (22–26 hr at 25°C after egg laying) of *E2/D17* were selected on the basis of their morphological features, and levels of *DE-cadherin* and *catenins* were compared to those of the wild type, which had two copies of *shg*⁺ (Fig. 8A). The *E2/D17* embryos of this age contained nearly 20% of the wild-type level of *DE-cadherin* protein expression. This abundance was confirmed in embryos homozygous for *Df(2R)D17* (Fig. 8B). These embryos were selectable at earlier stages (13–16 hr) because their whole morphology was in disarray. In immunostaining for *DE-cadherin*, the mutant embryos stained less brightly than wild-type siblings at stage 8, and they did not give signals at cell–cell junctions except for weak ones in the amnioserosa at stage 13. At later stages, no signals were obtained at cellular boundaries throughout the embryos (Fig. 2C). Thus, despite the presence of a certain level of maternally derived *DE-cadherin*, we could not clearly detect them at cell–cell contact sites within the range of the sensitivity of immunohistology.

The zygotic removal of *DE-cadherin* expression also reduced the expression of β -catenin. Two forms of *Arm*/ β -catenin are generated by alternative splicing (J. Loureiro and M. Peifer, pers. comm.). The larger isoform, which is thought to be involved in *Wg* signaling, is predominantly associated with *DE-cadherin* (Oda et al. 1994). Western blot analysis showed that the *shg* null mutation dramatically decreased the level of this large form (Fig. 8A,B, arrow). In sharp contrast, the amount of the smaller neural-specific isoform was not altered (Fig. 8A,B, arrowhead). As for *D α -catenin*, only a slight decrease in its total level was observed (Fig. 8A,B). This would be at least partly attributable to the strong expression of *D α -catenin* in axon tracks that were not affected by *shg* mutations (see below).

Images of embryos immunostained for *Arm* were consistent with the results of the Western analyses (Fig. 9A–D). In the wild-type embryo, in addition to the ubiquitous expression of membrane-associated *Arm* molecules, cells that received the *Wg* signal also accumulated *Arm* in the cytoplasm (Peifer et al. 1994), generating the well-known *Arm* transversal stripes (Fig. 9A). The *shg* null embryo displayed the normal *Arm* stripes until stage 9, but signals at both the stripes and interstripe regions started to decay at stage 10 (Fig. 9B). This alteration of the *Arm* patterns did not appear to affect epidermal cell fates, as shown by denticle patterns (Fig. 3B). In epithelial tissues of the wild-type embryo at later developmental stages, the apical zone of cell–cell boundaries (Fig. 9C), where *ZA* develops, was enriched with *Arm* (Peifer 1993). However, in every *shg* mutant embryo, these signals of *Arm* were greatly weakened not only in MTs and trachea but also in all the other epithelia (Fig. 9D). We could not detect any organized arrays of staining signals along intercellular junctions for *D α -catenin* either (Fig. 9E,F). Finally, the central nervous system (CNS) in the mutant embryo exhibited intense immunostaining signals for both *D α -catenin* and *Arm*, as in the wild type. Images for *D α -catenin* staining are shown in Figure 9, G and H.

Figure 6. Outgrowth and fusion of tracheal branches were blocked in *shg* mutants. All photographs are lateral views, and oriented with anterior to the left and ventral down. (A–H) Images of tracheal lumina, stained with no. 84, which were constructed by overlaying multiple optical sections. (A,B) Mid-stage 13 embryos of the wild type and *E2/D17*, respectively. In the wild type, DTs were about to fuse together. (C,D) Stage 14 embryos of the wild type and the deficiency, respectively. The DT fusion has already taken place at late stage 13 in the wild type. Arrows represent visceral branches, and arrowheads point to LTs. (E) Stage 14 deficiency embryo that was partially rescued by the expression of the *hsp-cadE* transgene. (F) Stage 14 *shg*² mutant. Arrows indicate examples of constrictions at the fusion points of DTs. Interruptions of tracts are labeled by asterisks (*). (G,H) Stage 16 wild-type and *shg*^{E17B} embryos, respectively. This *shg*^{E17B} mutant (H) is slightly younger than the normal embryo of G. In the mutant, every DT joint was pinched (arrows) and some LTs were not connected (asterisks). (I,J) Early stage 13 embryos of the wild type and the deficiency, respectively, that were double-stained for β -gal expression of a *breathless*-enhancer trap line (red) and *esg* (yellow or green). Tracheal tip cells expressed *esg* (arrowheads in I and J). Scale bar, 50 μ m.

Discussion

We presented evidence that the processes of dynamic epithelial reorganization require the zygotic expression of DE-cadherin, encoded by *shotgun* (*shg*). Elimination of the zygotic expression destroyed the MT and the ventral epidermis, and blocked distinct aspects of tracheal tree formation. A strong *shg* mutation was reported previously to affect the cell morphology of midgut epithelia, which are of endodermal origin (Tepass and Hartenstein 1994b). Assembled midgut epithelial cells remained round or cuboidal instead of becoming columnar. This phenotype is consistent with our finding of the role of DE-cadherin in epithelial reorganization processes.

The role of maternal DE-cadherin expression

Unexpectedly, most of the epithelial tissues were not severely disorganized by *shg* mutations. They maintained not only their mutual associations but also apicobasal polarity. Even after fragmentation of the MT, the mutant cells still preserved these two aspects of cellular characters. The zygotic null mutant contained a significant amount of maternally derived DE-cadherin, suggesting that this maternal pool may contribute to the maintenance of the general epithelial architecture. To test this possibility, we attempted to generate germ-line clones, but the obtained clones displayed blocked oogenesis [H. Oda, unpubl.]. For this reason, we were unable to

Figure 7. Tracheal development in *arm* and *wg* mutants. Lateral views oriented with anterior to the left and ventral down. Staining and image reconstruction were done as explained in Fig. 6. (A,B) *arm* null mutants (*arm*^{YD35}) of late stage 13 and early stage 16, respectively. Dorsal longitudinal trunks were totally missing (cf. A with Fig. 6C and B with Fig. 6C). Lateral trunks (arrowhead) did not fuse with each other in B. Arrows represent visceral branches. (C) Stage 16 *arm* hypomorph (*arm*^{H8.6}). Connections of dorsal and lateral trunks were incomplete (arrow and asterisk, respectively). (D) Stage 14 *wg* null mutant (*wg*^{CX4}). Many of the tracheal cells remained on the surface ectoderm (star), but a dorsal trunk was generated (arrow). In *arm*^{YD35}, no immunoreactivity of no. 84 could be detected on the embryonic surface. Scale bar, 50 μ m.

study the effect of the removal of maternal *DE*-cadherin on embryogenesis. The same type of experiment was done successfully by another group who employed weak alleles (Tepass et al., this issue). Those embryos produced only small patches of the cuticle over their whole body. This cuticle pattern strongly suggests that all of the cuticle-secreting epithelia, including the entire epidermis, were totally destroyed. This observation supports the idea that the maternal *DE*-cadherin plays an essential role in the initial formation and maintenance of epithelial tissues.

The above argument about the maternal contribution of *DE*-cadherin, however, appears inconsistent with our immunohistological observations of the zygotic *shg* mutants; that is, neither *DE*-cadherin nor its associated proteins, $\Delta\alpha$ -catenin and Arm, were highly concentrated at cell–cell boundaries in embryos at late stages, for example, after stage 13. Nevertheless, the results of the *shg* germ-line clones, together with those of the zygotic mutants, indicate that the drastically reduced levels of the *DE*-cadherin/catenin complex can still manage to maintain the epithelial architecture once it has been established. Perhaps, our immunohistological method was not sensitive enough for fully detecting the maternal molecules.

Previously we showed similar features in the expression patterns and in vitro activities of *DE*-cadherin and vertebrate E-cadherin, and considered *DE*-cadherin to be a *Drosophila* homolog of the vertebrate E-cadherin (Oda et al. 1994). The *shg* studies reinforce the notion that these two proteins appear to be equivalent in the sense of in vivo functions as well.

The role of DE-cadherin in dynamic epithelial rearrangements

How does the zygotic *DE*-cadherin control epithelial reorganization? During cell rearrangement within a tissue

like the MT, cells must cycle a rapid breakdown and a re-establishment of their mutual adhesion without creating intercellular breakages (Gumbiner 1992). Another type of dynamic cell adhesion is found in the ventral neurogenic region of the ectoderm, where epidermal precursors have to remake new contacts as soon as neuroblasts delaminate (Hartenstein and Campos-Ortega 1984). In the *shg* zygotic mutants, epithelial structures were broken down in both the MT and the neuroectoderm (this study; Tepass et al., this issue). These severe damages present a sharp contrast to the undisrupted tissues including the dorsal epidermis and the hindgut. The dorsal epidermis is morphogenetically less active than the ventral epidermis at the stage of neuroblast segregation, and so is the hindgut than the MT during the elongation of the latter. Maternal *DE*-cadherin molecules, detected in the ventral epidermis and also presumably present in the MTs, could not support the reorganization of these tissues. We therefore speculate that the zygotic *DE*-cadherin expression is critically required for the establishment of new cell–cell associations and that the maternal molecules cannot perform this task. It is conceivable that the pool of the maternal *DE*-cadherin is not sufficient in amount, and/or it cannot be recycled, to establish new cell–cell adhesions. That is, the maternal molecules may serve only for the maintenance of the already established cell–cell junctions, whereas the ones supplied by the zygotic expression are utilized for active cell repositioning.

In the absence of well-controlled intercellular adhesion systems, the activated cell motility would cause a disruption of epithelial monolayers. Adhesion molecules need to be delivered rapidly to appropriate positions to form new cell–cell contacts in a manner coordinated with the cell movement. Interaction of *DE*-cadherin with the actin-based cytoskeleton via catenins likely supports an efficient redistribution of the molecules on the plasma membrane. Also, detachment of cells must

Figure 8. Western blotting analyses of deficiency and *shg*² mutant embryos. (A,B) DE-cadherin (DE-cad), β -catenin/Arm, D α -catenin (D α -cat) in deficiency embryos (Df) were compared with those in wild-type Oregon R (WT). *E2/D17* embryos (22–26 hr) (A) or 13–16 hr *Df(2R)D17* homozygotes (B) were used. In B, besides the mature form of DE-cadherin (150 kD, short arrow), a larger precursor is seen. Two Arm isoforms are generated, an epithelial type (long arrow, 105–110 kD) and a smaller neural type (arrowhead, 82 kD) (J. Loureiro and M. Peifer, pers. comm.). D α -cat is 110 kD in its full size, and smaller bands seen are most likely degradation products. The 65-kD subunit (A subunit) of protein phosphatase 2A (PP2A_A; Shiomi et al. 1994) was used as a standard to compare the amounts of proteins loaded. In each lane, a whole lysate equivalent to ~15 embryos was loaded. Proteins in the deficiency embryos were more extractable, probably because of the elimination of most ventral cuticle. (C) Patterns of DE-cadherin in Oregon R (WT) and *shg*² homozygote (*shg*²). Degraded DE-cadherin molecules were detected in the *shg*² extract (arrowheads). A position of β -galactosidase (116 kD) is indicated by a bar in C.

be controlled during cell rearrangement processes. A study done by use of E-cadherin/ α -catenin fusion proteins suggests that catenins play a pivotal role in reversibility of intercellular adhesion (Nagafuchi et al. 1994). Therefore, cadherins seem to be ideal regulators of dynamic cell–cell adhesion. Recently one of the *Xenopus* cadherins was implicated in gastrulation movements (Lee and Gumbiner 1995); this finding appears to be consistent with ours on the *shg* zygotic mutants.

In normal MT organogenesis, cellular rearrangement follows the termination of cell proliferation. Although we could not determine exactly when the proliferation phase was over in the mutant organ, the disintegration process was observed at stages when cells were supposed to be still dividing in the wild type. In the normal embryo, MT cells start proliferation after almost all of the other ectodermal epithelia have finished cell division (for review, see Skaer 1993). During mitosis, intercellular junctions persist (Tepass and Hartenstein 1994a), and DE-cadherin and catenins stay at the apical positions of epithelial cell contacts (Woods and Bryant 1993; Harten-

stein et al. 1994; T. Uemura, unpubl.). The mutant epithelial sheet may not be able to stand sufficiently the mechanical stress brought about by rapid proliferation, resulting in a certain extent of epithelial disorganization. This initial rupture of cell contacts may be exaggerated once the cells acquire motility and undergo rearrangement.

DE-cadherin and Arm functions in the extension and fusion of tracheal branches

Tracheal tubular extension was partially blocked in the *shg* null mutant. A number of other gene products also play important roles in embryonic tracheal patterning; they include Breathless, a fibroblast growth factor (FGF) receptor homolog (Klambt et al. 1992), Thick veins, a receptor serine/threonine kinase (Affolter et al. 1994), and Drifter, a POU-domain transcription factor (Anderson et al. 1995). Compared with the phenotypes of mutants of these genes, the *shg* phenotypes are unique in that the defects were rather restricted to a subset of branches that perform fusion, that is, fusion branches (Manning and Krasnow 1993). Although cellular mechanisms for elaboration of individual branch extensions are not fully understood, such restricted effects of the *shg* mutations suggest that different mechanisms for tubular outgrowth are operative between the fusion branches and the other branches. For the elongation of the fusion branches, it is likely that cell rearrangement may be a major force, as is the case in MT development. In contrast, for the other branches, for example, visceral branches, mechanisms such as simple cell shape changes, which would not accompany cellular repositioning, might be essential. The tracheal epithelial structure was damaged much less seriously than that of the MTs. This could be attributable to possible involvement of multiple mechanisms, as described above, operating in the elongation processes.

Analysis of partial loss-of-function *shg* mutants revealed another DE-cadherin-dependent process in the tracheal fusion. In the weak allele *shg*^{E17B}, DTs were able to extend far enough so that their tracheal tip cells established physical contact with each other, and thus lumina were generated to connect flanking branches. However, widening of the tract diameter was blocked at every fusion point. To understand more definitively the roles of DE-cadherin in the fusion processes, we must clarify the cellular basis of the fusion, in particular, the behavior of the tip cells.

Besides the *shg* mutants, *arm* mutants also showed malformation of the fusion branches. This is not surprising superficially, because Arm and DE-cadherin are supposed to interact physically with each other in their function. However, the situation in the *arm* mutant is somewhat complicated. In the *arm* zygotic null mutant, DE-cadherin proteins were detectable at the apical side of cell boundaries in all epithelia, although the signal was weaker than that in wild-type siblings (data not shown). Those cadherin molecules probably form complexes with maternally derived Arm (Riggleman et al.

Figure 9. Distribution of catenin signals in the deficiency embryos (right column) and wild-type siblings (left column). (A–D) Arm staining of formaldehyde-fixed embryos. (E–H) $D\alpha$ -catenin staining of embryos fixed by the heat-methanol method (E, F) or by formaldehyde (G, H). (A, B) Stage 10 Arm stripes in ventrolateral views. (C–F) Dorsolateral views of stage 13/14 embryos. (MT) Malpighian tubules; (HG) hindgut; (DT) dorsal longitudinal trunks of the trachea tree. The arrowhead in F indicates a residual $D\alpha$ -catenin signal in the mutant MT that did not fall apart completely in this stage of the embryo. (G, H) Ventral views of the central nervous system in stage 14 wild-type and stage 15 deficiency embryos, respectively. Axon tracks in the deficiency embryo were stained as strongly as in the wild type, although the deficiency embryo had a couple of breaks in the longitudinal connectives and commissures (arrowheads). The deficiency embryos were a *Df(2R)E2* homozygote (B) and a transheterozygote of *E2/D17* (D, F, H). Confocal images were processed with identical parameters for A and B, C and D, E and F, and G and H. Anterior is to the left. Scale bar, 75 μ m in A and B, and 50 μ m in C–H.

1990), suggesting that they are functioning as adhesion machinery to a certain extent. Consistently, the *arm* null mutant exhibited normal extension of MTs as opposed to the *shg* mutant. Therefore, the tracheal malformation in the *arm* embryo cannot simply be explained by a reduction in the activity of the cadherin–catenin adhesion system. Arm is considered to work as a signal transducer downstream of Wg in patterning of the embryonic epidermis [for review, see Peifer 1995]. However, tracheal phenotypes in *arm* and *wg* mutants indicate that these two genes function, at least in part, separately in this tubulogenic morphogenesis.

Finally, it would be intriguing to ask whether *DE*-cadherin is involved in the Wg signaling cascade for segment polarity determination. The denticle patterns in the *shg* null mutant did not provide positive evidence for this possible linkage, although the loss of most ventral cuticles hindered careful analyses of the patterning. Weak-

ening of the overall Arm stripe pattern was observed in the *shg* null mutant. However, this reduction of the Arm level became apparent during stage 10, when most cells no longer require active Wg for stable *engrailed* (*en*) expression [Bejsovec and Martinez Arias 1991; Heemskerk et al. 1991]. The maternally derived *DE*-cadherin perhaps sustained the Arm level at the critical period for Wg signaling, and thus the effect of loss of *DE*-cadherin on segment polarity could not be examined. Further studies are necessary to disclose whether *DE*-cadherin is actively involved in the Wg pathway.

Materials and methods

Drosophila strains

Isolation of deficiency chromosomes in the 57B region is described elsewhere [Kraut and Campos-Ortega 1996]. Embryos from each line were stained with an antibody to *DE*-cadherin,

and we found that signals were undetectable in late homozygous *Df(2R)E2* embryos. *Df(2R)E2/+* and *Df(2R)D17/+* polytene chromosomes were then hybridized with DE-cadherin cDNA probes, and we confirmed that both deficiencies completely lacked *shg*. *Df(2R)D17*, *Df(2R)p113*, *shg²*, and *wg^{CX4}* were provided from the Bloomington Stock Center. *Df(2R)F36* and *Df(2R)PL3* were reported previously (O'Donnell et al. 1989).

Before isolation of *Df(2R)E2* and finding that *shg* encodes DE-cadherin, we tried to find candidates of *cadE* mutants among a collection of lethal strains that mapped within *Df(2R)D17*. For this purpose, we used a subset of the lethal lines that positioned within *Df(2R)D17* but outside all internal deficiencies like *Df(2R)F36* and *Df(2R)PL3*. These lethal strains were provided from Dr. Janis O'Donnell (University of Alabama, Tuscaloosa). Embryos of those individual lines were stained with the anti-DE-cadherin antibody; two lines, *l(2)E17B* and *l(2)SHN-1*, showed ectopic signals in axon tracks in the CNS. However, *cadE* mRNA was detected only in midline glial cells in these lines as in the wild type (T. Usui and T. Uemura, unpubl.). We did not study further the molecular basis of the ectopic protein distribution in these lines. By complementation tests with *shg²*, we found that those two were allelic to *shg*, so they were renamed *shg^{E17B}* and *shg^{SHN-1}*, respectively.

In FlyBase, we found that the Berkeley *Drosophila* Genome Project identified three strains with PZ-element insertions into 57B13-14: *l(2)03050*, *l(2)07206*, and *l(2)10469* (FlyBase 1994; Spradling et al. 1995; Berkeley *Drosophila* Genome Project, pers. comm.). These three lines were provided from the Bloomington Stock Center. *l(2)10469* did not complement the three *shg* alleles that we described above, and we renamed this line *shg^{P10469}*. The PZ-element was mobilized by $\Delta 2-3$ (Robertson et al. 1988). Adult escapers homozygous for *shg^{P10469}* showed a patterning defect along the anterior wing margin (Y. Kataoka and T. Uemura, unpubl.).

arm^{YD35} and *arm^{H8.6}* were provided by Dr. Mark Peifer (University of North Carolina, Chapel Hill). *arm^{H8.6}* is a temperature-sensitive allele (Klingensmith et al. 1989), and embryos homozygous for it were incubated at 25°C for tracheal analyses. The following enhancer trap lines were used: A154.2M3 (Bellen et al. 1989) to study MTs and 6-81a (Klambt et al. 1992) for tracheal cells.

Isolation of genomic DNA clones of the DE-cadherin gene

We isolated phage clones from an Oregon-R genomic library (L. Jan and Y.N. Jan, University of California, San Francisco) using DE-cadherin cDNA clones (Oda et al. 1994). About 20 kb of genomic DNA in total was isolated, and we roughly determined positions of exons by hybridizing various cDNA fragments to the genomic clones, comparison of restrictions sites, and partial genomic sequencing. The position of the *shg^{P10469}* insertion was determined by sequencing the plasmid rescue fragment.

Antibodies and Western blotting

Rat antibodies to DE-cadherin (DCAD1 and DCAD2), $\Delta\alpha$ -catenin (DCAT1), Esg, and protein phosphatase 2A_A were generated previously (Oda et al. 1993, 1994; Fuse et al. 1994; Shiomi et al. 1994). DCAD1 was used for Western analyses and DCAD2 for immunostaining embryos. Other antibodies used were as follows: 7A1 (mouse anti-Arm; Peifer 1993), Cq4 (mouse anti-CRB; Tepass and Knust 1993), no. 84 (guinea pig tracheal-specific antibody; Klambt et al. 1992), DA1B6 (mouse anti-fasciclin III; Gauger et al. 1987), no. 243 (rabbit anti- β -Heavy-spectrin antibody; Thomas and Kiehart 1994), and rabbit anti- β -galactosidase (Cappel). To prepare samples for Western blots, we homog-

enized dechorionated embryos in a 1× Laemmli sample buffer containing 1 mM phenylmethylsulfonyl fluoride, sonicated the homogenate, and immediately boiled it for 3–4 min. To detect signals in Western blots, we employed an ECL kit (Amersham).

Observation of embryos

For immunostaining, embryos were usually fixed in 3.5% formaldehyde in 0.1 M phosphate buffer (pH 7.4), or a protocol of heat methanol fixation was followed (U. Tepass, pers. comm.). The heat methanol fix was employed for β -Heavy-spectrin (Fig. 4H,I) and $\Delta\alpha$ -catenin (Fig. 9E,F) stainings. $\Delta\alpha$ -catenin is concentrated at the ZA in heat-fixed embryos (U. Tepass, pers. comm.). We did not employ any fix solutions containing EDTA or EGTA, because cadherins are degraded easily under Ca^{2+} -free conditions. Signals were detected by use of an ABC Elite kit (Vectastain) or by fluorescence labeling (Oda et al. 1994). A combination of FITC and Texas Red was employed for double labeling, and embryos were viewed with a Zeiss laser scanning confocal microscope. MTs in live embryos were observed with Normarski optics except that a condenser for dark-field microscopy was used.

Heat shock experiments

A 5-kb fragment of the DE-cadherin cDNA was isolated from pRmHa3-DECH (Oda et al. 1994) and inserted into pCaSpeR-hs (Thummel et al. 1988). The resultant plasmid, phs-DECH, was microinjected into *w¹¹¹⁸*; $\Delta 2-3$ *TM3 Sb/Dr* (Robertson et al. 1988) to produce transgenic flies. Two established lines were used for rescue experiments: DECH 1-1-2, which has phs-DECH inserted into the X chromosome, and DECH 2-3 (with insertion into the third chromosome). For the rescue experiments we collected males and virgin females, each of which was heterozygous for *Df* or for *shg²* and carried one copy of the DECH chromosome. Those males and females were crossed to each other. Embryos were collected, aged for 3.5–5 hr at 25°C, and heat-shocked for 30 min in a 37°C water bath. They were then aged to stage 13–14, to stage 16 for observation of tracheal development, or to the end of embryogenesis for examination of cuticles and MTs. Embryos of the deficiency or *shg²* strains without phs-DECH were heat-shocked in parallel for the control. As for DECH 1-1-2 and DECH 2-3, the overexpression was confirmed by RNA in situ hybridization, Western blot analysis, and immunostaining. Both chromosomes exhibited similar levels of the DE-cadherin induction and equally rescued the embryos from the mutant phenotypes.

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