

Regulatory mechanisms required for DE-cadherin function in cell migration and other types of adhesion

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Cadherin-mediated adhesion can be regulated at many levels, as demonstrated by detailed analysis in cell lines. We have investigated the requirements for *Drosophila melanogaster* epithelial (DE) cadherin regulation in vivo. Investigating *D. melanogaster* oogenesis as a model system allowed the dissection of DE-cadherin function in several types of adhesion: cell sorting, cell positioning, epithelial integrity, and the cadherin-dependent process of border cell migration. We generated multiple fusions between DE-cadherin

and α -catenin as well as point-mutated β -catenin and analyzed their ability to support these types of adhesion. We found that (1) although linking DE-cadherin to α -catenin is essential, regulation of the link is not required in any of these types of adhesion; (2) β -catenin is required only to link DE-cadherin to α -catenin; and (3) the cytoplasmic domain of DE-cadherin has an additional specific function for the invasive migration of border cells, which is conserved to other cadherins. The nature of this additional function is discussed.

Introduction

Classic cadherins are major mediators of cell–cell adhesion. Their extracellular domain mediates calcium-dependent homophilic cell–cell adhesion, whereas their highly conserved intracellular domain is linked to the actin cytoskeleton. The link to the cytoskeleton is essential for adhesion and is provided by catenins (mainly α - and β -catenin). The COOH-terminal domain of the cadherin intracellular domain binds to β -catenin, which, in turn, binds to α -catenin; α -catenin then directly and indirectly interacts with actin filaments (Nagafuchi and Takeichi, 1989; Ozawa et al., 1990; Jou et al., 1995; Knudsen et al., 1995; Rimm et al., 1995; Watabe-Uchida et al., 1998; Weiss et al., 1998).

During development and in adult organisms, cadherins mediate different types of cell–cell adhesion. For instance, they are required to maintain stable adhesion between epithelial cells (Larue et al., 1994; Riethmacher et al., 1995; Tepass et al., 1996; Uemura et al., 1996) but can also be used by migrating cells to adhere to a cellular substratum (Letourneau et al., 1990; Barami et al., 1994; Hazan et al., 2000; Li et al., 2001). Cell migration is thought to require dynamic regulation of adhesion. A number of mechanisms that may regulate cadherin-mediated adhesion have been suggested from tissue culture experiments. In particular, because linking cadherin to actin filaments is essential for adhesion, modulation of this link has been proposed

to regulate adhesion strength. For example, mouse L cells expressing an epithelial (E) cadherin/ α -catenin fusion protein can adhere to each other in a way that is similar to L cells expressing E-cadherin but seem to be unable to regulate adhesion (Nagafuchi et al., 1994). Multiple mechanisms, in particular the phosphorylation of cadherin and catenins, could regulate the link between cadherin and α -catenin either at the level of cadherin/ β -catenin interaction or of β -catenin/ α -catenin interaction (Balsamo et al., 1998; Kuroda et al., 1998; Rosato et al., 1998; Lickert et al., 2000; Bek and Kemler, 2002).

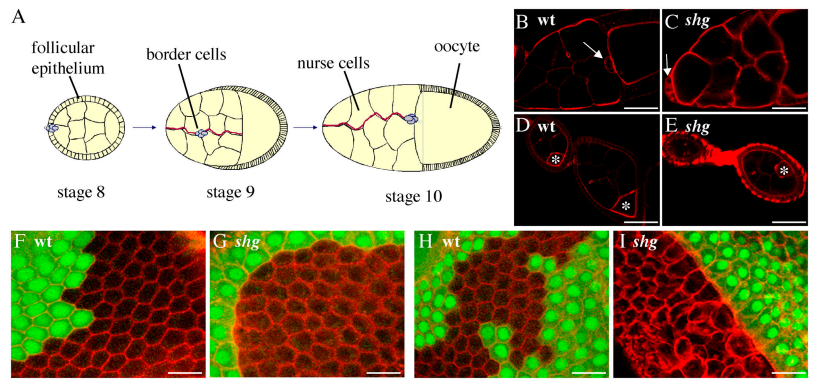
In this study, we use *Drosophila melanogaster* epithelial (DE) cadherin–mediated adhesion during *D. melanogaster* oogenesis as a model to study adhesion regulation in different types of adhesion in vivo. DE-cadherin is a classic cadherin that is encoded by the *shotgun* (*shg*) gene and is associated with junctional complexes in epithelia (Oda et al., 1994). During *D. melanogaster* oogenesis, DE-cadherin is also required for the invasive migration of border cells. Border cells are a group of about eight somatic follicle cells that delaminate from the follicular epithelium, invade the germ line cluster, and migrate to the oocyte (Fig. 1 A). Both border and nurse cells express DE-cadherin, and a lack of DE-cadherin in either cell type blocks migration (Fig. 1, B and C; Oda et al., 1997; Niewiadomska et al., 1999). This indicates that border cells adhere to the nurse cell substratum through homophilic DE-cadherin interaction and that this adhesion is essential for migration. For border cells to translocate, DE-cadherin–mediated adhesion may need to be effectively regulated to generate strong

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Abbreviations used in this paper: arm, armadillo; DE, *Drosophila* epithelial; FL, full length; JM, juxtamembrane domain; shg, shotgun; UAS, upstream activating sequence.

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Figure 1. DE-cadherin function during oogenesis. (A) Schematic representation of border cell migration. (B) Wild-type stage 10 egg chamber; border cells (arrow) have reached the oocyte. (C) Border cells (arrow) do not migrate when border or nurse cells are mutant for *shg* (DE-cadherin). (D) The oocyte (asterisks) is located in the posterior of wild-type egg chambers. (E) Oocyte mislocalization is often observed when follicle or nurse cells are *shg* mutant. (F and G) Follicle cell sorting; stage 9 egg chambers. Compared with control clones (F), *shg* mutant clones sort away from wild-type cells to form a smooth interface (G). (H and I) Follicular epithelium integrity; stage 10 egg chambers. *shg* mutant clones lose epithelial integrity (I, compare cell shape with that of the wild-type clone in H). (B–I) Phalloidin (red) stains F-actin. (F–I) Mutant clones are indicated by an absence of GFP (green). Bars (B and C), 80 μ m; (D and E) 50 μ m; (F–I) 20 μ m.



adhesion at the front as well as the release of adhesion in the back. To investigate DE-cadherin regulation in this context, we generated DE-cadherin mutant variants and analyzed their ability to replace the endogenous protein and support border cell migration. We also analyzed their ability to mediate other types of adhesion during *D. melanogaster* oogenesis. In *D. melanogaster* egg chambers, DE-cadherin is required to maintain epithelial integrity in follicle cells (Fig. 1, H and I; Tanentzapf et al., 2000). Moreover, it mediates differential cell affinities in the follicular epithelium as well as during oocyte positioning in egg chambers (Fig. 1, D–G; Godt and Tepass, 1998; González-Reyes and St. Johnston, 1998). Analyzing each of these processes allows us to distinguish general DE-cadherin function and regulation from migration-specific ones.

Results

Interaction with β -catenin is essential for DE-cadherin function

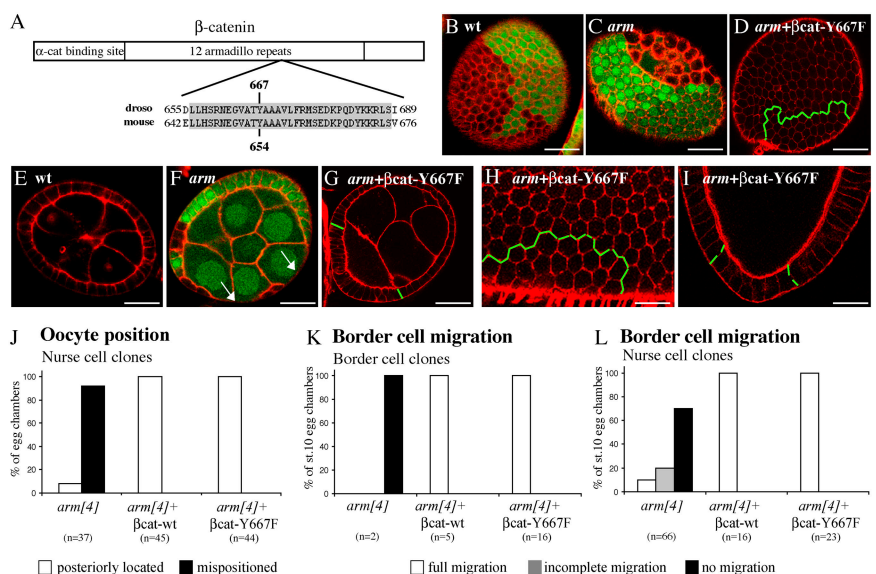
Mutations in *armadillo* (*arm*), which is the single *D. melanogaster* β -catenin gene, give rise to phenotypes that show its requirement in cadherin-dependent adhesion. During oogenesis, *arm*-null mutant germ line clones cause oocyte mispositioning

(Fig. 2 J; Peifer et al., 1993; Godt and Tepass, 1998; González-Reyes and St. Johnston, 1998). Also, in the follicular epithelium, *arm* mutant cells sort away from wild-type cells and lose their epithelial integrity (Fig. 2, C and F; González-Reyes and St. Johnston, 1998; Tanentzapf et al., 2000). *arm*-null mutant follicle cells lose their epithelial integrity earlier than *shg* mutant cells, which is likely a result of the presence of both *Drosophila* neural (DN) and DE-cadherin in early follicle cells (Tanentzapf et al., 2000). Also, *arm* mutant border cells do not migrate (Fig. 2 K). In nurse cells, lack of β -catenin strongly inhibits migration without completely blocking it (Fig. 2 L; Peifer et al., 1993). The linkage to the cytoskeleton via β -catenin may be less critical for cadherin to function as substratum than in the actively migrating cell itself. Finally, a mutant form of DE-cadherin that is unable to bind β -catenin is also not able to mediate adhesion during *D. melanogaster* oogenesis (Pacquelet et al., 2003), confirming that interaction with β -catenin is essential for DE-cadherin function.

Phosphorylation of β -catenin tyrosine 667 is not critical for function

Because linking cadherin to actin filaments through catenins is essential for adhesion, modulation of this link would be a

Figure 2. Analysis of β -catenin-Y667F function. (A) Schematic representation of β -catenin. Sequences flanking Y654 in mouse β -catenin and the corresponding Y667 in *D. melanogaster* β -catenin (*arm*) are indicated. (B–I) *arm* mutant clones sort away from wild-type cells and display epithelial integrity defects. (C and F) Stage 6 egg chambers; arrows indicate resulting discontinuity in the epithelium. Compare with wild-type clones in B and E. β -catenin-Y667F rescues both sorting (D and H) and follicular epithelium integrity (D, G, and I) even at late stages of oogenesis (H and I, stage 10 egg chambers). Phalloidin (red) stains F-actin, and mutant clones are indicated by an absence of GFP (green) or by the green line (below the line in D and H and between the lines in G and I). (J–L) Oocyte positioning and border cell migration in *arm* mutant clones expressing the indicated β -catenin transgenes. In nurse cell clones (J and L), β -catenin-wt and β -catenin-Y667F were expressed from an *arm* promoter. In follicle and border cell clones (D, G–I, and K), they were expressed as UAS transgenes with the MARCM system. Bars (B–G), 30 μ m; (H and I) 20 μ m.



mechanism for regulating adhesion. Phosphorylation of a conserved tyrosine residue of β -catenin (tyrosine 654 of mouse β -catenin; Fig. 2 A) can induce β -catenin to dissociate from cadherin, hence resulting in a decrease of adhesion (Behrens et al., 1993; Balsamo et al., 1998; Rosato et al., 1998; Roura et al., 1999; Rhee et al., 2002). To test whether phosphorylation of this conserved tyrosine was essential (e.g., for adhesion down-regulation during border cell migration), we mutated the corresponding tyrosine in *D. melanogaster* β -catenin to phenylalanine (Fig. 2 A, Y667F). We then analyzed β -catenin–Y667F biological activity by testing its ability to substitute for endogenous β -catenin. Endogenous β -catenin was removed from specific cells by generating homozygous *arm*-null mutant clones in the context of transgenic flies expressing wild-type β -catenin or β -catenin–Y667F ubiquitously (under control of the *arm* promoter) or expressing it selectively in mutant cells using the MARCM system (see Flies).

Both β -catenin and β -catenin–Y667F rescued cell sorting and loss of epithelial integrity, which were caused by the lack of endogenous β -catenin in follicle cells, as well as oocyte mispositioning, which was caused by the lack of endogenous β -catenin in germ line cells (Fig. 2, D and G–J). β -Catenin and β -catenin–Y667F also fully rescued migration defects that were caused by the absence of endogenous β -catenin in border and nurse cells (Fig. 2, K and L). Thus, regulation of adhesion through β -catenin tyrosine Y667 phosphorylation is not essential for any β -catenin–dependent process during *D. melanogaster* oogenesis, including border cell migration.

DE-cadherin Δ Cyt/ α -catenin is not able to support border cell migration

Multiple other mechanisms that are involved in regulating adhesion by modulating the link between cadherin and catenins have been described previously (Kuroda et al., 1998; Kwon et al., 2000; Bek and Kemler, 2002). To simultaneously abolish all of these possible regulatory mechanisms, constitutive interaction between DE-cadherin and catenins was provided by fusing DE-cadherin directly to β - or α -catenin. DE-cadherin/ β -catenin fusion proteins were found to be non-functional and to behave as dominant negative in vivo (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200506131/DC1>). Therefore, they were not used further.

DE-cadherin Δ Cyt/ α -catenin was obtained by fusing full-length (FL) α -catenin directly after the transmembrane domain of DE-cadherin (Fig. 3 A). This fusion protein should link DE-cadherin to the cytoskeleton but prevent any regulation of the link between DE-cadherin and β -catenin as well as between β - and α -catenin. Functionality in vivo was analyzed by the ability to provide DE-cadherin function to cells lacking endogenous *shg* (DE-cadherin). Both wild-type DE-cadherin and DE-cadherin Δ Cyt/ α -catenin–expressing transgenes rescued the phenotypes that were observed in *shg* mutant follicle cell clones (follicle cell sorting, loss of epithelial integrity, and oocyte mispositioning; Fig. 3, E and G). Thus, DE-cadherin Δ Cyt/ α -catenin can substitute for endogenous DE-cadherin in follicle cells. This shows that it is able to mediate productive adhesion. Wild-type DE-cadherin fully rescued migration defects of

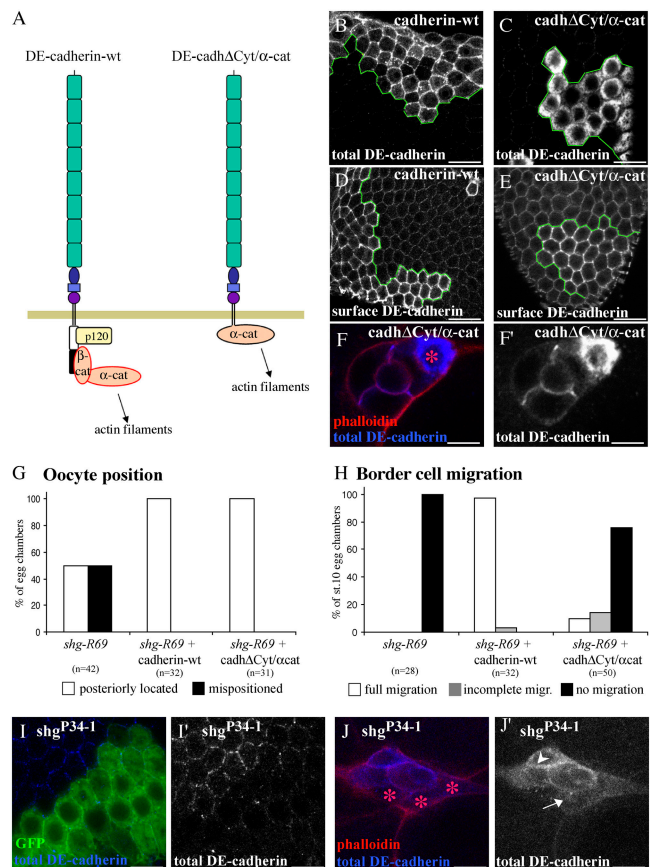


Figure 3. Activity of DE-cadherin with α -catenin in place of the cytoplasmic domain (DE-cadherin Δ Cyt/ α -catenin). (A) Schematic representation of wild-type DE-cadherin and DE-cadherin Δ Cyt/ α -catenin. (B–F') Expression of wild-type DE-cadherin (B and D) and DE-cadherin Δ Cyt/ α -catenin (C, E, and F) in *shg*-null mutant (*shg*^{R69}) follicle (B–E) and border cells (F). (B–E) Edge of the mutant clone (cells with increased staining) is indicated by the green line. (F) One border cell (asterisk) is mutant. (B, C, and F) All DE-cadherin is detected. (D and E) Only surface DE-cadherin is detected. (G) Oocyte positioning when follicle cells are *shg*-null mutant and express the indicated transgenes. (H) Border cell migration in *shg*-null mutant border cells expressing the indicated transgenes. Three independent transgenic lines were tested for DE-cadherin Δ Cyt/ α -catenin. (I–J') DE-cadherin levels in *shg*^{P34-1} mutant follicle (I) and border cell (J) clones. (I) Mutant cells are indicated by the presence of GFP (green). (J) Three border cells (asterisks) were mutant. Compare DE-cadherin staining between adjacent wild-type cells (arrowhead) with staining between adjacent mutant cells (arrow). (F, I, and J) Phalloidin (red) stains F-actin and DE-cadherin is in blue. Bars (B–E, I, and I'), 20 μ m; (F, F', J, and J') 10 μ m.

shg mutant border cells (Fig. 3 H). In contrast, DE-cadherin Δ Cyt/ α -catenin showed almost no rescue ability in border cells (Fig. 3 H).

To understand why DE-cadherin Δ Cyt/ α -catenin could not support border cell migration, we first checked expression levels and subcellular localization in follicle and border cells. In all experiments, both wild-type DE-cadherin and fusion proteins were somewhat overexpressed. This did not affect the function of wild-type DE-cadherin. DE-cadherin Δ Cyt/ α -catenin was expressed at high levels and showed a strong intracellular accumulation (Fig. 3, C and F). Wild-type DE-cadherin also accumulated intracellularly (Fig. 3 B), but at a lower level. This intracellular accumulation is likely to correspond to retention in the ER (Fig. S2, available at <http://www.jcb.org/cgi/content/full/>

jcb.200506131/DC1). A surface-labeling protocol was used to check that DE-cadherin Δ Cyt/ α -catenin was present at cell membranes. There was slightly more DE-cadherin Δ Cyt/ α -catenin at the membrane in *shg* mutant follicle cells than endogenous DE-cadherin in wild-type cells (Fig. 3 E). Because border cells are located inside egg chambers, surface labeling could not be performed on these cells. We infer from surface levels in follicle cells that DE-cadherin Δ Cyt/ α -catenin membrane levels in border cells should be close to endogenous levels. Moreover, a *shg* hypomorphic allele (*shg*^{P34-1}; Tepass et al., 1996) that clearly reduces DE-cadherin levels (Fig. 3, I and J) only weakly inhibited border cell migration (5% of stage 10 egg chambers show slight migration delays when border cells are mutant). Thus, DE-cadherin levels in border cells can be significantly lowered without significantly affecting border cell migration. Altogether, this indicates that the phenotype of DE-cadherin Δ Cyt/ α -catenin is not caused by insufficient DE-cadherin Δ Cyt/ α -catenin surface levels but reflects a specific inability of this fusion protein to fulfill DE-cadherin function in border cells.

Regulation of the link between DE-cadherin and α -catenin is not required for border cell migration

The inability of DE-cadherin Δ Cyt/ α -catenin to support border cell migration could be a result of the covalent fusion of α -catenin to DE-cadherin, which would indicate that a regulation of the link between DE-cadherin and α -catenin is required for border cell migration. Alternatively, the absence of rescue could be a result of the lack of a sequence in DE-cadherin cytoplasmic domain that is required for DE-cadherin function in border cells. To distinguish between these two possibilities, FL DE-cadherin/ α -catenin was generated in which the FL cytoplasmic domain of DE-cadherin was retained and α -catenin fused at the COOH terminus (Fig. 4 A). Similarly to DE-cadherin Δ Cyt/ α -catenin, DE-cadherin-FL/ α -catenin prevents any regulation of the link between DE-cadherin and α -catenin but still carries all the information that may be contained in the DE-cadherin cytoplasmic domain. DE-cadherin-FL/ α -catenin was expressed efficiently in *shg* mutant follicle cells (Fig. 4 B), accumulated at the plasma membrane (Fig. 4 C), and, to some extent, accumulated inside the cells. DE-cadherin-FL/ α -catenin rescued *shg* phenotypes such as follicle cell sorting (Fig. 4 B), loss of epithelial integrity (Fig. 4 B), and oocyte mispositioning (Fig. S3 A, available at <http://www.jcb.org/cgi/content/full/jcb.200506131/DC1>). DE-cadherin-FL/ α -catenin also fully rescued the migration defects of *shg* mutant border cells (Fig. 4 G).

The complete rescue that was obtained with DE-cadherin-FL/ α -catenin suggests that regulating the link between DE-cadherin and α -catenin is not required during border cell migration. However, DE-cadherin-FL/ α -catenin still contains the β -catenin-binding domain and, therefore, might primarily be linked to actin via endogenous β - and α -catenin. To address this point, we tested whether DE-cadherin-FL/ α -catenin was functional in the absence of endogenous β -catenin by expressing it in *arm*-null mutant clones. In the absence of β -catenin, DE-cadherin-FL/ α -catenin subcellular localization looked relatively normal

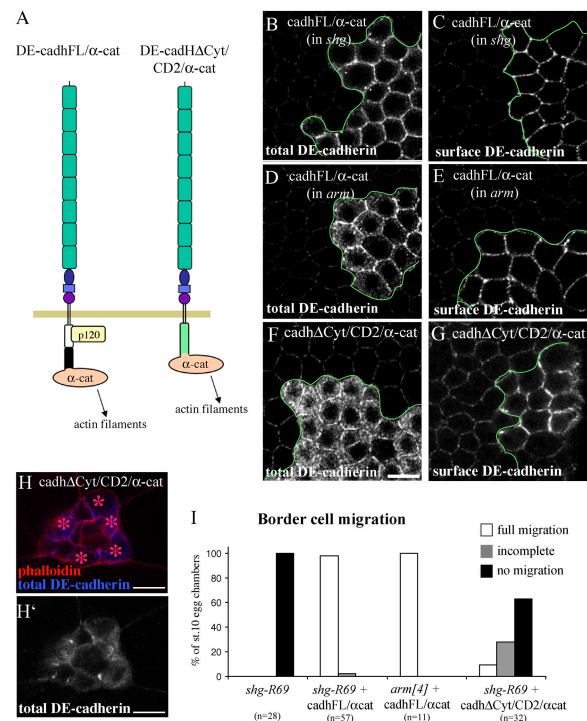


Figure 4. Role of the cytoplasmic tail of DE-cadherin. (A) Schematic representation of DE-cadherin-FL/ α -catenin and DE-cadherin Δ Cyt/CD2/ α -catenin. (B–H) Expression of DE-cadherin-FL/ α -catenin (B–E) and DE-cadherin Δ Cyt/CD2/ α -catenin (F–H) in *shg* mutant follicle cells (B, C, F, and G) and border cells (H) or in *arm* mutant follicle cells (D and E). (B–G) Mutant cells are to the right of and/or below the green line. (H) Five border cells (asterisks) are *shg* mutant and express UAS–DE-cadherin Δ Cyt/CD2/ α -catenin. (B, D, F, and H) All DE-cadherin is detected. (C, E, and G) Only surface DE-cadherin is detected. (H) Phalloidin (red) stains F-actin and DE-cadherin is in blue. (I) Border cell migration in *shg*- or *arm*-null mutant border cell clones expressing the indicated transgenes. Bars (F), 20 μ m; (H and I) 10 μ m.

(Fig. 4, compare D and E with B and C), although minor alterations in protein localization cannot be ruled out. DE-cadherin-FL/ α -catenin fully rescued *arm* mutant clones with respect to oocyte positioning (20/20 were normal), sorting, and integrity of follicular epithelium (Fig. 4, D and E) as well as border cell migration (Fig. 4 G). This shows that DE-cadherin-FL/ α -catenin is fully functional in border cells and other cells even in the absence of endogenous β -catenin. Thus, in all of these contexts, β -catenin does not have any other essential role for DE-cadherin function than linking it to α -catenin.

In conclusion, the link between DE-cadherin and α -catenin does not need to be regulated for proper DE-cadherin function during border cell migration, nor does it need to be regulated in any other type of DE-cadherin function that was examined.

Lack of the cytoplasmic domain explains DE-cadherin Δ Cyt/ α -catenin inability to support border cell migration

DE-cadherin Δ Cyt/ α -catenin and DE-cadherin-FL/ α -catenin are very different in their ability to support border cell migration. At least three explanations could account for this difference: (1) In DE-cadherin Δ Cyt/ α -catenin, α -catenin could be too close to the membrane, creating conformational problems

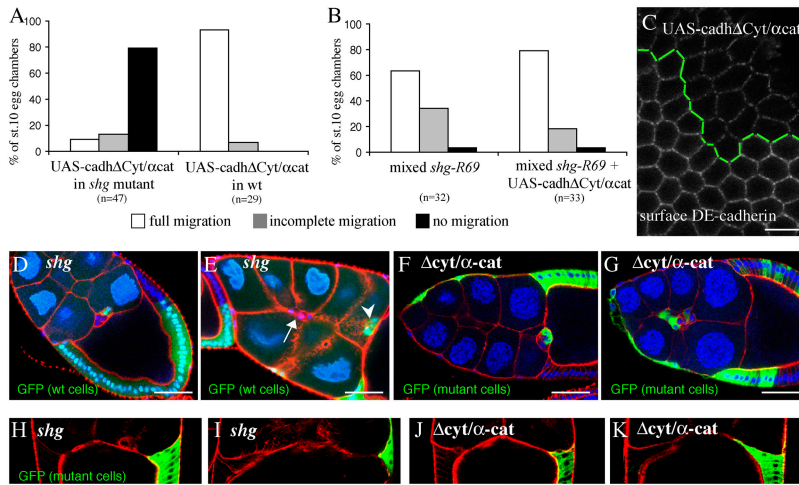


Figure 5. Further analysis of DE-cadherin Δ Cyt/ α -catenin phenotypes. (A) Migration defects of border cells expressing DE-cadherin Δ Cyt/ α -catenin in *shg* mutant or wild-type background. (B) Migration defects of mixed border cell clusters. In each scored cluster, half of the border cells are wild type and half are as indicated (D–G). (C) DE-cadherin surface levels in wild-type cells expressing DE-cadherin Δ Cyt/ α -catenin (below the green line). (D and E) Mixed border cell cluster, wild-type, and *shg* mutant cells. At stage 10, the full cluster has reached the oocyte (D), or *shg* mutant cells (E, arrow) are split from wild-type cells (E, arrowhead; incomplete migration in B). (F and G) Mixed border cell cluster, wild-type, and *shg* mutant cells expressing DE-cadherin Δ Cyt/ α -catenin. At stage 10, the cluster has reached the oocyte (F) or is delayed (G, incomplete migration). (H–K) Centripetal cell migration and direct comparison of mutant (green) with wild-type cells. *shg* mutant cells (H, early; and I, late) show defects; *shg* mutant cells expressing DE-cadherin Δ Cyt/ α -catenin (J, early; and K, late) show no defect ($n = 23$). Phalloidin (red) stains F-actin and DAPI (blue) stains nuclei. GFP (green) marks wild-type cells in D and E and mutant cells in F–K. Bars (C), 20 μ m; (D–G) 80 μ m.

that could result in the poor functionality of DE-cadherin Δ Cyt/ α -catenin. (2) The pronounced intracellular accumulation of DE-cadherin Δ Cyt/ α -catenin could have negative effects on migration. (3) A signal that is present in the cytoplasmic tail of DE-cadherin and DE-cadherin-FL/ α -catenin but is absent in DE-cadherin Δ Cyt/ α -catenin could be required for DE-cadherin function during migration. To discriminate between these possibilities, an additional fusion protein was generated. DE-cadherin Δ Cyt/CD2/ α -catenin is composed of the extracellular and transmembrane domain of DE-cadherin (DE-cadherin Δ Cyt), the cytoplasmic domain of an unrelated protein, CD2, and FL α -catenin (Fig. 4 A).

In follicle and border cell *shg* mutant clones, DE-cadherin Δ Cyt/CD2/ α -catenin was expressed efficiently (Fig. 4, F and H), and the cell surface level was slightly higher than endogenous DE-cadherin levels (Fig. 4 G). By quantifying the staining (see Immunostainings), we found that intracellular accumulation of this fusion was fourfold lower than that of DE-cadherin Δ Cyt/ α -catenin and twice that of DE-cadherin-FL/ α -catenin. DE-cadherin Δ Cyt/CD2/ α -catenin could fully replace endogenous DE-cadherin in follicle cell sorting, epithelial integrity (Fig. 4, F and G), and oocyte positioning (Fig. S3 A) but not in border cells (Fig. 4 I). Migration defects of border cells expressing only DE-cadherin Δ Cyt/CD2/ α -catenin were similar to those observed with DE-cadherin Δ Cyt/ α -catenin. Thus, the inability of DE-cadherin Δ Cyt/ α -catenin to support border cell migration appears not to be caused by the short distance between DE-cadherin and α -catenin. It is also unlikely to be caused by intracellular protein accumulation, as the intracellular accumulation of DE-cadherin Δ Cyt/CD2/ α -catenin was more similar to that of DE-cadherin-FL/ α -catenin (which is functional). Consistently, the effect of DE-cadherin Δ Cyt/ α -catenin in border cells is not dominant (see the next section). Thus, migration defects that were observed with DE-cadherin Δ Cyt/ α -catenin are most likely caused by the absence of a signal that is present in the DE-cadherin cytoplasmic domain. Furthermore, this signal appears to be specifically required for DE-cadherin function during border cell migration.

Why is the cytoplasmic domain of DE-cadherin required for border cell migration?

For border cells to migrate, two properties of DE-cadherin are likely to be important: (1) to generate sufficient adhesion so that traction forces exerted by the cytoskeleton can efficiently pull the cells forward (force-bearing adhesion); and (2) to allow down-regulation of adhesion such that cells can let go at the rear, detaching first from follicle cells and then from nurse cells along the migratory path. The inability of DE-cadherin Δ Cyt/ α -catenin to function during border cell migration could be caused by either an inability to generate enough adhesive strength for cell translocation or an inability for adhesion to be down-regulated.

If the migration defects that were observed in border cells expressing only DE-cadherin Δ Cyt/ α -catenin were caused by a lack of adhesion down-regulation and, hence, an excess of adhesion, one would expect DE-cadherin Δ Cyt/ α -catenin to behave in a dominant way. Border cell migration should be perturbed when DE-cadherin Δ Cyt/ α -catenin is expressed in the presence of endogenous DE-cadherin and when half of the border cells in a cluster express only DE-cadherin Δ Cyt/ α -catenin, whereas the other half express endogenous DE-cadherin. When DE-cadherin Δ Cyt/ α -catenin was expressed in wild-type egg chambers in a manner similar to what was performed in the *shg* rescue experiment, it was targeted to the cell membrane (Fig. 5 C) but gave rise to only very mild migration defects (Fig. 5 A). In mixed border cell clusters in which half of the cells were wild type and the other half were *shg* mutant cells expressing DE-cadherin Δ Cyt/ α -catenin, we observed mild delays (Fig. 5 B). This phenotype was similar to what was observed in *shg* mixed border cell clusters (half of the border cells were wild type and half were *shg* mutant; Fig. 5 B). In this latter situation, it is thought that *shg* mutant cells are pulled by the wild-type cells, explaining why most border cell clusters migrate normally (Niewiadomska et al., 1999). Thus, DE-cadherin Δ Cyt/ α -catenin does not have a prominent dominant inhibitory effect during border cell migration, indicating that its inability to

support migration is not simply caused by a lack of adhesion down-regulation.

We observed two differences in the behavior of mixed border cell clusters depending on whether *shg* mutant cells express no DE-cadherin or express DE-cadherin Δ Cyt/ α -catenin. When *shg* mixed clones showed migration defects, border cell clusters were split; wild-type border cells had migrated normally, whereas *shg* border cells had been left behind (Fig. 5 E). Although DE-cadherin is not strictly required for adhesion between adjacent border cells, this likely reflects a contribution of DE-cadherin to adhesion between border cells. Also, border cell clusters were either composed of only mutant cells, only wild-type cells, or half wild-type cells and half *shg* mutant cells with no intermediate ratio (e.g., no clusters with one *shg* mutant cell). This phenomenon is specific for *shg* mutant clones and may be based on sorting between wild-type and *shg* mutant follicle cells (González-Reyes and St. Johnston, 1998). In a border cell cluster, each of the two central polar cells is expected to interact directly with half of the outer border cells. Wild-type or *shg* polar cells would preferentially adhere to and, therefore, recruit wild-type or *shg* outer border cells, respectively. In the presence of DE-cadherin Δ Cyt/ α -catenin, mixed border cell clusters were never split (Fig. 5 G), and all possible ratios of wild-type to mutant cells were observed. These observations show that DE-cadherin Δ Cyt/ α -catenin is functional and mediates basic adhesion in border cells, supporting the conclusion that the inability of this fusion to rescue border cell migration reflects the loss of a migration-specific function.

To determine whether the cytoplasmic domain of DE-cadherin would be generally required for somatic cells to invade the germ line cluster, we looked at centripetal cells. Centripetal follicle cells move as a sheet in between the oocyte and nurse cells at stage 10 and, in the absence of DE-cadherin, mostly fail to migrate and eventually round up (Fig. 5, H and I; Niewiadomska et al., 1999). Contrary to the situation in border cells, we saw no defect in *shg* mutant centripetal cells expressing DE-cadherin Δ Cyt/ α -catenin (40 egg chambers were analyzed). Even the timing seemed normal when we compared mutant cells directly with wild-type cells of the same egg chamber (Fig. 5, J and K). Thus, the requirement in border cells is rather specific, possibly reflecting the more absolute requirement for DE-cadherin in this process (Niewiadomska et al., 1999).

What is the signal missing in DE-cadherin Δ Cyt/ α -catenin?

We sought to define more precisely which part of the cytoplasmic domain is specifically required for DE-cadherin function during border cell migration. We first tested whether the most COOH-terminal domain of DE-cadherin contained a contributing sequence by generating the intermediate fusion DE-cadherin Δ β / α -catenin (Fig. 6, A and B). In follicle and border cell *shg* mutant clones, DE-cadherin Δ β / α -catenin was expressed at high levels and accumulated inside the cells (Fig. 6, C and E) as well as at the cell surface (Fig. 6 D) in amounts similar to those observed with DE-cadherin Δ Cyt/ α -catenin. DE-cadherin Δ β / α -catenin fully rescued *shg* follicle cell sorting and epithelial integrity (Fig. 6 D) as well as oocyte mispositioning

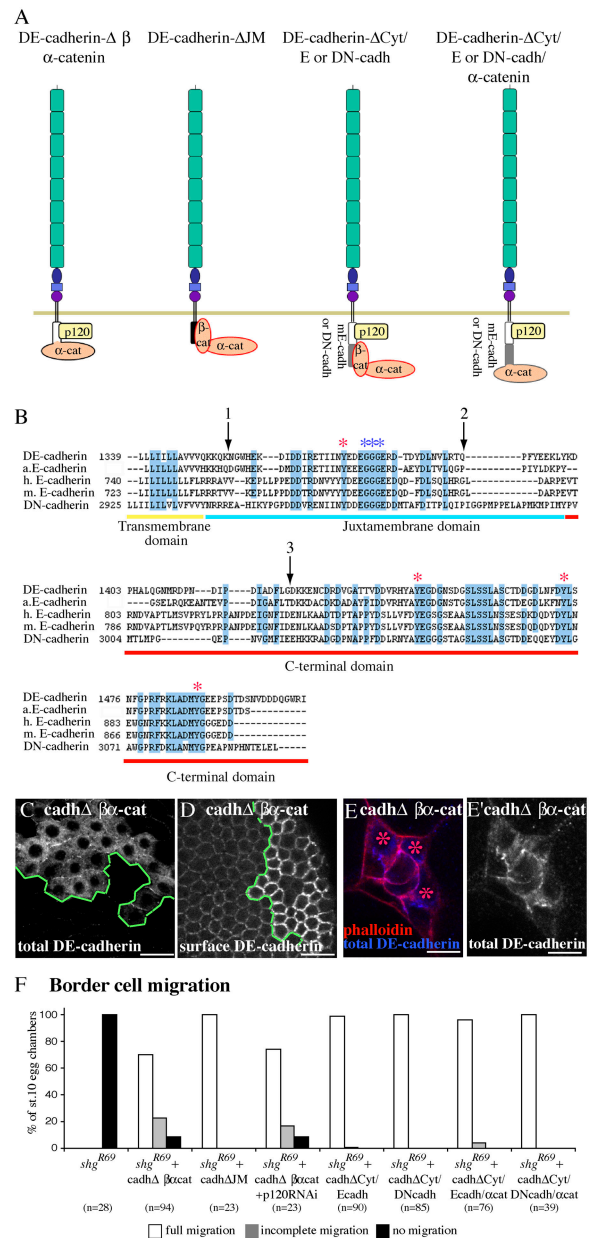


Figure 6. Molecular dissection of DE-cadherin cytoplasmic domain. (A) Schematic representation of proteins that were analyzed. (B) Alignment of *D. melanogaster*, *Anopheles*, human and mouse E-cadherin, and *D. melanogaster* N-cadherin transmembrane and cytoplasmic domains. In DE-cadherin Δ Cyt/ α -catenin and DE-cadherin Δ β / α -catenin, α -catenin was fused at arrows 1 and 3, respectively. In DE-cadherin Δ JM, the region between arrows 1 and 2 was deleted. Blue asterisks indicate p120 catenin-binding sites. Red asterisks indicate conserved tyrosines mutated in DE-cadherin-4YF. The limit between JM and COOH-terminal domain was drawn after studies on mammal E-cadherin. (C–E') Expression of DE-cadherin Δ β / α -catenin in *shg*-null mutant follicle (C and D) and border cells (E). (C and D) Mutant cells are above and to the right of the green line. (E) Three border cells (asterisks) are *shg* mutant and express DE-cadherin Δ β / α -catenin. (C and E) All DE-cadherin is detected. (D) Only surface DE-cadherin is detected. (E) Phalloidin (red) stains F-actin and DE-cadherin is in blue. (F) Border cell migration in *shg* mutant border cell clones expressing the indicated UAS transgenes. Bars (C and D), 20 μ m; (E and E') 10 μ m.

(Fig. S3 A). In border cells, DE-cadherin Δ β / α -catenin partially rescued *shg* migration defects (Fig. 6 F). This indicates that the most COOH-terminal domain of DE-cadherin, although it is

not strictly essential, contributes to efficient border cell migration. As border cells expressing DE-cadherin-FL/ α -catenin in the absence of β -catenin migrate normally, this appears to be independent of binding to β -catenin.

Next, we tested whether the DE-cadherin juxtamembrane domain (JM) played an essential role. A mutant form of DE-cadherin lacking the JM (Fig. 6, A and B, DE-cadherin Δ JM) was generated. In *shg* mutant border cells, DE-cadherin Δ JM fully rescued the migration (Fig. 6 F). It also rescued all other *shg* phenotypes during oogenesis (Fig. S3, A–C and F) as well as lethality caused by the absence of endogenous DE-cadherin in the embryo (Fig. S3 G). Thus, DE-cadherin Δ JM on its own does not play an essential role during development.

The JM contains a p120 catenin-binding site, and *D. melanogaster* has one p120 catenin protein that has a supporting but nonessential role in cell adhesion (Myer et al., 2003). To test whether it could have a redundant function with a sequence located in the COOH-terminal domain, we coexpressed DE-cadherin $\Delta\beta$ / α -catenin and a p120 catenin RNA interference transgene that severely reduces the expression of p120 catenin (Pacquelet et al., 2003) in *shg* mutant border cells. However, knocking down p120 catenin did not increase the migration defects that were observed with DE-cadherin $\Delta\beta$ / α -catenin (Fig. 6 F).

Four tyrosine residues are located in DE-cadherin cytoplasmic domain at positions that are conserved in mammalian cadherins: one in the JM and three in the COOH-terminal domain. Because tyrosine phosphorylation of cadherin has been implicated in adhesion regulation (Fujita et al., 2002), we mutated the four conserved tyrosine residues to phenylalanines to obtain DE-cadherin-4YF (Fig. 6 B). DE-cadherin-4YF could substitute for endogenous DE-cadherin in border cells (Fig. S3 E). It also rescued all *shg* phenotypes during oogenesis (Fig. S3, A, B, D, and F) as well as lethality that was caused by a lack of endogenous DE-cadherin in the embryo (Fig. S3 G). Thus, phosphorylation of the four conserved tyrosine residues that are present in DE-cadherin cytoplasmic tail is not required for any essential function of DE-cadherin.

DE-cadherin Δ JM lacks aa 1355–1392 (Fig. 6 B, between arrows 1 and 2), whereas amino acids that are downstream of aa 1425 are lacking in DE-cadherin $\Delta\beta$ / α -catenin (Fig. 6 B, arrow 3). Thus, it is possible that an essential signal missing in DE-cadherin Δ Cyt/ α -catenin is located between aa 1393 and 1424 (Fig. 6 B, between arrows 2 and 3). However, this linker region is very poorly conserved between cadherin molecules (Fig. 6 B). To determine whether interactions performed by the DE-cadherin cytoplasmic tail during migration could be satisfied by other cadherin molecules, we analyzed four additional fusion proteins (Fig. 6 A). These had the cytoplasmic tail of either mouse E-cadherin or *D. melanogaster* neural (N) cadherin in place of the DE-cadherin cytoplasmic domain or were fused to α -catenin (as in DE-cadherin-FL/ α -catenin). Each of these fusions rescued border cell migration completely (Fig. 6 F), indicating that this additional function of the cytoplasmic tail is conserved. From a biological perspective, the existence of this migration-related function in an N-cadherin molecule seems logical, as N-cadherin has been linked to migration and even

invasion in multiple systems (Letourneau et al., 1990; Barami et al., 1994; Hazan et al., 2000; Li et al., 2001).

Discussion

Classic cadherin proteins have multiple essential roles during animal development both in keeping tissues/epithelia intact and in allowing dynamic cell rearrangements. One dramatic example of the latter is the invasive migration of border cells during oogenesis, for which DE-cadherin is essential (Niewiadomska et al., 1999). We have investigated which features of DE-cadherin are required for migration and compared them with features that are required more generally for other adhesion functions. Cadherin proteins are well conserved from fly to man; the cytoplasmic domain, in particular, is well conserved, and it interacts with the cytoskeleton. Therefore, we focused our *in vivo* genetic analyses on dissecting the functions of DE-cadherin cytoplasmic domain. In the type of *in vivo* replacement experiments that we performed, we can make clear conclusions about what is and is not required under physiological conditions. This is the strength of the analysis, and we think it is important to further our understanding of the much-studied cadherin molecules. Generally speaking, we cannot exclude the idea that a type of regulation that is not genetically required does, in fact, occur under normal conditions and contribute somewhat to regulation (e.g., to make the system more robust).

No requirement for regulation of the links between DE-cadherin and α -catenin

We initially focused on a conserved tyrosine of β -catenin, the phosphorylation of which may induce β -catenin to dissociate from cadherin, resulting in a decrease of adhesion. We found that this conserved tyrosine (and, hence, its phosphorylation) is not essential even during border cell migration. We do not exclude the idea that phosphorylation of this tyrosine residue happens or that it may induce some dissociation of β -catenin from DE-cadherin. What our results show is that such phosphorylation is not an essential mechanism for adhesion regulation in any of the tested types of cadherin-dependent adhesion *in vivo*. Significant emphasis has been put in the literature on the putative regulatory role of this conserved tyrosine of β -catenin (Behrens et al., 1993; Balsamo et al., 1998; Rosato et al., 1998; Roura et al., 1999; Rhee et al., 2002). However, much of this emphasis is based on correlations between β -catenin tyrosine phosphorylation and adhesion down-regulation. It is not clear whether β -catenin phosphorylation is really the cause of adhesion down-regulation. In addition, the tyrosine kinase Src causes a decrease of adhesion in L cells expressing the fusion protein E-cadherin/ α -catenin (Takeda et al., 1995). Thus, Src-induced adhesion down-regulation can be independent of β -catenin phosphorylation. Therefore, the ability to regulate adhesion without phosphorylating β -catenin tyrosine may be more general.

Next, we found that neither the link between DE-cadherin and β -catenin nor that between β - and α -catenin need be regulated at all for DE-cadherin function *in vivo*. A fusion between DE-cadherin-FL and α -catenin fully substituted for endogenous

DE-cadherin during oogenesis even in the absence of endogenous β -catenin. We were surprised to find that there is no need to regulate the link between DE-cadherin and α -catenin, as earlier studies using similar fusion proteins had concluded that regulation was required for mouse E-cadherin to support “intercellular migration” (Nagafuchi et al., 1994). There are two main differences with our study that can explain this discrepancy. First, the previous study did not fuse α -catenin to E-cadherin-FL but fused to a truncated E-cadherin (analogous to our DE-cadherin $\Delta\beta/\alpha$ -catenin). As we have found in this study, this not only affects the ability to regulate the link to α -catenin but also removes additional functionality from cadherin. It was not directly investigated in Nagafuchi et al. (1994) whether the defects were caused by β -catenin regulation as proposed. Second, different cell types were analyzed; the previous study overexpressed E-cadherin in mouse fibroblasts that normally have very little of the protein, whereas we investigated cells that normally depend on DE-cadherin for biological function.

It is possible that the link between DE-cadherin and the actin cytoskeleton does need to be regulated but that it occurs downstream of α -catenin. More studies of α -catenin and of how its interactions are regulated will be of interest, in particular in a physiological context. Alternatively, regulation of adhesion may primarily occur by the turning over of DE-cadherin and/or DE-cadherin complexes via endocytosis. A Cbl-related E3 ligase called Hakai has been identified as a specific regulator of mammalian E-cadherin endocytosis (Fujita et al., 2002). It is recruited to specific phosphorylated tyrosines on E-cadherin. We found no evidence that the homologous *D. melanogaster* protein (CG10263) affects DE-cadherin or border cell migration (unpublished data), and the key docking tyrosines are not conserved. However, other regulators may play an analogous role. Finally, adhesive strength could be regulated by lateral clustering of cadherin complexes; for example, by the binding of additional regulatory proteins to the intracellular domain.

The full functionality of DE-cadherin-FL/ α -catenin in the absence of β -catenin also indicates that β -catenin has no essential adhesive function other than linking DE-cadherin to α -catenin. Based on the abnormal localization of various DE-cadherin mutants, it had been proposed that β -catenin was required for proper translocation of cadherin to the plasma membrane (Chen et al., 1999). However, the relatively normal subcellular localization of DE-cadherin-FL/ α -catenin that was observed in the absence of β -catenin suggests that this is not generally the case. It remains possible that β -catenin also contributes to modifying cadherin localization in *D. melanogaster* cells, but in a more subtle, nonessential way. Our study suggests that parts of the cadherin tail that bind β -catenin may also have β -catenin-independent functions. This would complicate the interpretation of how modified cadherin molecules behave unless it is also investigated by β -catenin loss-of-function experiments.

Specific requirement for the cytoplasmic domain of DE-cadherin in migration

In contrast with DE-cadherin-FL/ α -catenin, a fusion protein between DE-cadherin and α -catenin lacking the DE-cadherin cytoplasmic tail (DE-cadherin Δ Cyt/ α -catenin) could not substi-

tute for DE-cadherin during border cell migration. It was targeted to the cell surface and was functional in all other contexts. This indicates that the DE-cadherin cytoplasmic tail has a specific function during invasive migration in addition to the basic β -catenin/ α -catenin linkage. The function could not be provided by an unrelated cytoplasmic linker (CD2) but could be provided by the corresponding region from mouse E-cadherin or *D. melanogaster* N-cadherin. Most likely, one or more interactions that are specific to cadherin tails have a critical function in this context. These results raise two questions: (1) why is DE-cadherin tail specifically important for border cell migration and (2) what is the molecular nature of the required function?

With regard to the specific requirement in border cells, we need to consider the role of DE-cadherin in their migration. Given the absolute requirement for this particular cell–cell interaction to achieve invasive border cell movement (Niewiadomska et al., 1999), it is likely to be force bearing. DE-cadherin-mediated adhesion between the front of border cells and the attachment point on nurse cells needs to be strong enough to allow border cells to pull themselves into the compact germ line tissue. As the border cell cluster initiates migration using a long, slender cellular extension (Fulga and Rørth, 2002), the local force application at the tip may be quite high. As an illustration of the forces involved, we found that mutant border cells with impaired cortical cytoskeleton will break apart when they attempt to invade, whereas other follicle cells (including centripetal cells) with the same defect appear to be relatively normal (Somogyi and Rørth, 2004). We suggest that the DE-cadherin tail may be required to allow a build-up of sufficiently strong adhesion to withstand forces that are involved in migration.

Another important aspect of adhesion during cell movement is that it may need to be effectively down-regulated at the rear of the cells to allow cell translocation along the substrate. Our experiments indicated that the primary defect for DE-cadherin Δ Cyt/ α -catenin in border cells was not a lack of down-regulation; in other words, it was not caused by an excess of adhesion. However, an inability of DE-cadherin Δ Cyt/ α -catenin to provide sufficient adhesion for migration as discussed above could mask possible additional (migration specific) defects of the fusion protein such as the ability to be down-regulated.

The molecular nature of the DE-cadherin tail requirement in migration is in need of further investigation. The function did not simply map to any previously known signal or interaction, suggesting involvement of a novel interaction and/or a redundancy of interactions. The DE-cadherin $\Delta\beta/\alpha$ -catenin fusion results indicate that the most COOH-terminal domain contributes to DE-cadherin function in border cells independently of β -catenin binding. However, this domain is not essential on its own nor when coexpressed with p120 catenin RNA interference constructs, indicating that additional important signals are located in the more proximal region of the DE-cadherin cytoplasmic domain. A mutant form of *Xenopus laevis* C-cadherin lacking the 94 proximal amino acids of its cytoplasmic domain can mediate some adhesion but is unable to support strong adhesion (Yap et al., 1998). This seems to be caused by its inability to form lateral clusters (Yap et al., 1998). Similarly, an absence of the proximal region in DE-cadherin Δ -

Cyt/ α -catenin could prevent its clustering and, thereby, prevent adhesion strengthening.

In conclusion, our structure/function analysis of DE-cadherin in different types of cell adhesion has given new information about cadherin regulation in vivo. Several previously defined potential points of regulation that were established through detailed work in tissue culture were found not to be essential for functionality in vivo. The cytoplasmic tail of cadherin was found to have a unique role in the demanding process of invasive cell migration, possibly through a novel interaction.

Materials and methods

Cloning

β -Catenin cDNA (obtained from Expressed Sequence Tag LD23131) was subcloned in pBS (XhoI). In β -catenin-Y667F, tyrosine 667 was mutated to phenylalanine by replacing ACATACGCCGCC with ACATTCGCCGCC (creates a NotI site). pCaSpeR-arm was obtained by subcloning the arm promoter (from pCaSpeR-armLacZ; Vincent et al., 1994) in pCaSpeR-4 (EcoRI-Asp718). pCaSpeR-arm- β -catenin-wt was obtained by cloning β -catenin cDNA in pCaSpeR-arm (SpeI-SnaBI-StuI). β -Catenin-Y667F was subcloned in pCaSpeR-arm by replacing the SpeI-NheI fragment of β -catenin in pCaSpeR-arm- β -catenin-wt. β -Catenin and β -catenin-Y667F were subcloned in pUASp2 (Asp718; pUASp [Rørth, 1998] with modified polylinker).

DE-cadherin cDNA was provided by B. Sanson (University of Cambridge, Cambridge, UK). For DE-cadherin Δ Cyt/ α -catenin, DE-cadherin Δ β / α -catenin, DE-cadherin Δ Cyt/ β -catenin, and DE-cadherin Δ β / β -catenin fusions, α -catenin cDNA (obtained from Expressed Sequence Tag LD07767) and β -catenin cDNA were cloned in frame with DE-cadherin Δ Cyt and DE-cadherin Δ β (Pacquelet et al., 2003). An XbaI site was inserted between the two cDNAs. For DE-cadherin Δ FL/ α -catenin fusion, α -catenin cDNA was cloned in frame with DE-cadherin just before the DE-cadherin stop codon, and a linker (five glycine or six lysine) was added between DE-cadherin and α -catenin. For DE-cadherin Δ Cyt/CD2/ α -catenin, DE-cadherin Δ Cyt was cloned in frame with the cytoplasmic domain of rat CD2 (aa 233–344; cDNA provided by S. Cohen, EMBL, Heidelberg, Germany) and FL α -catenin. For DE-cadherin Δ Cyt/E-cadherin/ α -catenin and DE-cadherin Δ Cyt/DN-cadherin/ α -catenin, the following was fused in frame to DE-cadherin Δ Cyt: the cytoplasmic domains of mouse E-cadherin (aa 741–884; sequence at fusion KKQRVREPLL; cDNA provided by M. Takeichi, RIKEN Center for Developmental Biology, Kobe, Japan) or DN-cadherin (aa 2944–3097; sequence at fusion KKQRAHIKYP; cDNA provided by T. Uemura, Kyoto University, Kyoto, Japan) followed by a five-glycine linker and FL α -catenin. For DE-cadherin Δ Cyt/E-cadherin and DE-cadherin Δ Cyt/DN-cadherin, a stop codon was placed after the same E- or DN-cadherin sequences instead of α -catenin sequences. All DE-cadherin fusion constructs were cloned into UASp2 (UASp with an altered polylinker) and analyzed in transgenic animals.

In DE-cadherin Δ JM, bp 4749–4862 were deleted. For DE-cadherin-4YF, four tyrosines (aa 1372, 1447, 1473, and 1488) were mutated to phenylalanines. Tyrosine Y1372 was mutated by replacing AATTAC with GAATTC (creates an EcoRI site). For Y1447, Y1473, and Y1488, TAC was replaced by TTC. DE-cadherin-4YF was subcloned into pCaSpeR-tubulin (Asp718 + NotI; plasmid provided by S. Cohen), and DE-cadherin Δ JM was subcloned in pCaSpeR-tubulin and pUASp (Asp718 + NotI).

Flies

Rescue experiments with upstream activating sequence (UAS)- β -catenin and UAS-DE-cadherin transgenes were performed with the MARCM system (Lee and Luo, 1999). Flies of the following genotypes were generated: *arm[4]FRT19A/tubGal80,hsFLP,FRT19A*; *UASCD8-GFP/+*; *tubGal4/UAS- β -catenin* and *hsFLP,UASCD8-GFP; FRT42Dshg^{R69}/FRT42DtubGal80,hsFLP*; and *tubGal4/UAS-DE-cadherin*. In the absence of mitotic recombination, Gal80 inhibits Gal4; after recombination, *arm* or *shg* mutant cells lack Gal4, and Gal4 is active and drives the expression of UAS-GFP and UAS- β -catenin or UAS-DE-cadherin transgenes. For rescue experiments with *arm- β -catenin* and tubulin-DE-cadherin transgenes, flies of the following genotypes were generated: *armFRT19A/ubiGFP,FRT19A*; *hsFLP; arm- β -catenin/+* and *hsFLP; FRT42shg/FRT42ubiGFP*; and *tubulin-DE-cadherin/+*. To express UAS-DE-cadherin Δ Cyt/ α -catenin in wild-type egg chambers, flies of the following geno-

type were generated: *hsFLP,UASCD8-GFP; FRT42D/FRT42DtubGal80,hsFLP*; and *tubGal4/UAS-DE-cadherin*. Clones were generated by heat-shocking flies as larvae or adults at least 4 d before analysis.

Flies were provided by the following individuals: *shg^{R69}* flies from U. Tepass (University of Toronto, Toronto, Canada); *shg³⁴* flies from A. Gonzalez-Reyes (Instituto de Parasitologia y Biomedicina, Granada, Spain); protein disulfide isomerase GFP flies from A. Ephrussi (EMBL); and UAS-GFP-FYVE flies by M. Gonzalez-Gaitan (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany).

Immunostainings

Ovaries were fixed with 4% PFA. Primary antibodies were rat DCAD2 (1:100; provided by H. Oda, JT Biohistory Research Hall, Takatsuki, Japan; Oda et al., 1994) and Golgi marker (1:100; Calbiochem; provided by A. Ephrussi, EMBL). Fluorescent secondary antibodies (Jackson ImmunoResearch Laboratories) and rhodamin-phalloidin (Molecular Probes) were used at 1:200 and 1:500, respectively, and 1 μ g/ml DAPI was used. All images were captured using confocal microscopy (model SP2; Leica). For quantification of cytoplasmic accumulation, nonsaturating settings were used, and cytoplasmic cadherin levels in clones were normalized to total cadherin in adjacent wild-type cells and were compared between samples (measured with National Institutes of Health image). To specifically stain plasma membrane-associated DE-cadherin, ovaries were dissected in ice-cold Grace's medium and were incubated for 1 h in primary antibody (DCAD2; 1:20 in PBS) at 4°C. After several washes in PBS at 4°C, they were fixed with 4% PFA in PBS for 20 min and were stained with secondary antibodies based on protocols described previously (Strigini and Cohen, 2000).

Online supplemental material

Fig. S1 shows that DE-cadherin/ β -catenin fusion proteins are not functional when expressed in vivo. Fig. S2 shows markers for different subcellular compartments in follicle cells. Fig. S3 shows the quantification of phenotypes for additional DE-cadherin mutants and fusion proteins that are mentioned in the text. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200506131/DC1>.

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