Cadherin Cad99C is required for normal microvilli morphology in *Drosophila* follicle cells

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Summary

Microvilli are actin-filled membranous extensions common to epithelial cells. Several proteins have been identified that localize to microvilli. However, most of these proteins are dispensable for the normal morphogenesis of microvilli. Here, we show by immunoelectron microscopy that the non-classical cadherin Cad99C localizes to microvilli of *Drosophila* ovarian follicle cells. Loss of Cad99C function leads to disorganized and abnormal follicle cell microvilli. Conversely, overexpression of Cad99C in follicle cells results in large bundles of microvilli. Furthermore, altered microvilli morphology correlates with defects in the assembly of the vitelline membrane, an extracellular layer secreted by follicle cells that is part of the eggshell. Finally, we provide evidence that Cad99C is the homolog of vertebrate protocadherin 15. Mutations in the gene encoding protocadherin 15 lead to the disorganization of stereocilia, which are microvilli-derived extensions of cochlear hair cells, and deafness (Usher syndrome type 1F). Our data suggest an essential role for Cad99C in microvilli morphogenesis that is important for follicle cell function. Furthermore, these results indicate that insects and vertebrates use related cadherins to organize microvilli-like cellular extensions.

Supplementary material available online at
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Key words: *Drosophila*, Microvilli, Vitelline membrane, Cadherin, Cad99C, Protocadherin 15

Introduction

Microvilli are extensions of the apical plasma membrane that are common to epithelial cells. Intestinal epithelial cells, for example, display numerous closely spaced microvilli on their apical surface. This tightly packed array of microvilli, known as the brush border, greatly increases the cell-surface area available for nutrient absorption (reviewed by Heintzelman and Mooseker, 1992). Microvilli contain a bundle of crosslinked actin filaments at their center that extend from the plasma membrane to the cell cortex. Microvillar proteins, especially those of the intestinal brush border, have been identified. Among these are the actin-crosslinking proteins villin, fimbrin and espin, as well as the motor protein myosin-1A (Bartles et al., 1998; Bretscher and Weber, 1978; Bretscher and Weber, 1980; Matsudaira and Burgess, 1979; Mooseker et al., 1980). Expression of villin and espin in tissue culture cells promotes the formation and elongation of microvilli, indicating that these proteins can play a role in the biogenesis of microvilli (Franck et al., 1990; Friederich et al., 1989; Loomis et al., 2003). Mice lacking myosin-1a display defects in the morphology and organization of brush border microvilli (Tyska et al., 2005). However, a normal-sized microvillar brush border can be assembled in the intestine of mice with defects in villin (Ferrary et al., 1999; Pinson et al., 1998). Thus, it seems that not all microvillar proteins are essential for microvilli morphogenesis. To understand the morphogenesis and function of microvilli better, it will be important to identify additional microvillar proteins and to test their requirement for the formation of microvilli.

We have used *Drosophila melanogaster* ovaries to study the morphogenesis of microvilli. The ovary is composed of approximately 16-20 ovarioles, each containing a chain of egg chambers proceeding through 14 stages from the germarium to the oviduct (Spradling, 1993). Each egg chamber consists of 16 germline cells, one oocyte and 15 nurse cells, encapsulated by a monolayer of somatic, epithelial follicle cells. During stage 9, the majority of follicle cells move towards the posterior of the egg chamber, forming a columnar epithelium covering the oocyte. The remaining follicle cells stretch to cover the nurse cells. During stage 10, follicle cells covering the oocyte display on their apical surface numerous microvilli approximately 1 μm in length (Mahowald, 1972; Mahowald and Kambysellis, 1980). These microvilli closely approach the surface of the oocyte and interdigitate with microvilli protruding from the oocyte. Gap junctions have been reported between microvilli of follicle cells and the oocyte, suggesting communication through small molecules among these cells (Mahowald and Kambysellis, 1980).

During stages 8-10, follicle cells surrounding the oocyte secrete vitelline membrane proteins from their apical surface that are needed, in conjunction with the subsequently secreted chorion proteins, to build the protective eggshell (reviewed by Waring, 2000). Initially, vitelline membrane proteins aggregate and form vitelline bodies located in between the microvilli (Mahowald, 1972; Mahowald and Kambysellis, 1980; Trougakos et al., 2001). In stage 11 of oogenesis, follicle cell microvilli then shorten and the vitelline bodies coalesce into a continuous layer of vitelline membrane, which is important for

the formation of a normal eggshell. The precise role of the follicle cell microvilli during the process of oogenesis is not known.

Cad99C is a non-classical member of the cadherin superfamily of Ca^{2+}-dependent cell adhesion molecules and contains 11 cadherin repeats in its extracellular region and a consensus site for a class I PDZ (PSD-95, Dlg, ZO-1) domain-binding site at its cytoplasmic C-terminus (Schlichting et al., 2005). In general, cadherins provide molecular links between plasma membranes (and in some cases between plasma membranes and extracellular matrix) through homophilic or heterophilic binding of the extracellular cadherin repeats on adjacent plasma membranes and the binding of their intracellular region to cytoskeleton-associated proteins (Gumbiner, 2005; Patel et al., 2003). In this way, cadherins mediate physical adhesion and/or cell-to-cell signaling (Wheelock and Johnson, 2003). Cadherins are involved in various processes, including the maintenance of epithelial integrity and cell polarity, cell sorting, growth control, synaptic specificity and tissue morphogenesis (Angst et al., 2001; Gumbiner, 2005; Salinas and Price, 2005; Tepass et al., 2000; Yagi and Takeichi, 2000). We generated flies mutant for *Cad99C* and reported that, whereas adult homozygous mutant *Cad99C* flies are viable, female mutant flies are sterile (Schlichting et al., 2005). Here, we have analyzed the role of Cad99C during oogenesis. We show that Cad99C is required for the normal assembly of the vitelline membrane and the integrity of the eggshell. We further find that Cad99C localizes to the microvilli of follicle cells and that, in the absence of Cad99C, follicle cell microvilli are disorganized and abnormal. Conversely, overexpression of Cad99C in follicle cells results in large bundles of microvilli. Finally, we provide evidence that Cad99C is the homolog of vertebrate protocadherin 15, a protein required for the proper organization of cochlea hair cell stereocilia, suggesting that insects and vertebrates use related cadherins to organize microvilli-like cellular extensions.

Results

Cad99C is required for the integrity of the eggshell

To begin to address the role of Cad99C during oogenesis, we analyzed the eggs laid by mated *Cad99C* mutant flies. We previously isolated four independent *Cad99C* mutant alleles (Fig. 1A). Western blot analysis, using an anti-Cad99C antiserum, failed to detect Cad99C in extracts from ovaries of any of the four mutant fly lines (Fig. 1B). In contrast with wild-type flies, or heterozygous mutant *Cad99C* flies, flies homozygous for any of the four *Cad99C* alleles laid eggs that spontaneously collapsed after deposition and that did not develop into larvae (data not shown). A similar phenotype has been reported for mutants with defective vitelline membranes (e.g. Savant and Waring, 1989). To test whether the vitelline membrane was affected in eggs laid by *Cad99C* mutant flies, we assessed the permeability of the eggs to the vital dye Neutral Red. Eggs in which the outer chorion layer has been experimentally removed are normally impermeable to small molecules such as Neutral Red, apparently due to the lipid wax layer covering the vitelline membrane (Margaritis et al., 1980; Papassideri et al., 1993). Defects in the vitelline membrane are thought to disrupt this lipid wax layer, thereby leading indirectly to the permeability of the eggs to Neutral Red. Fewer than 3% of eggs laid by control heterozygous *Cad99C* mutant

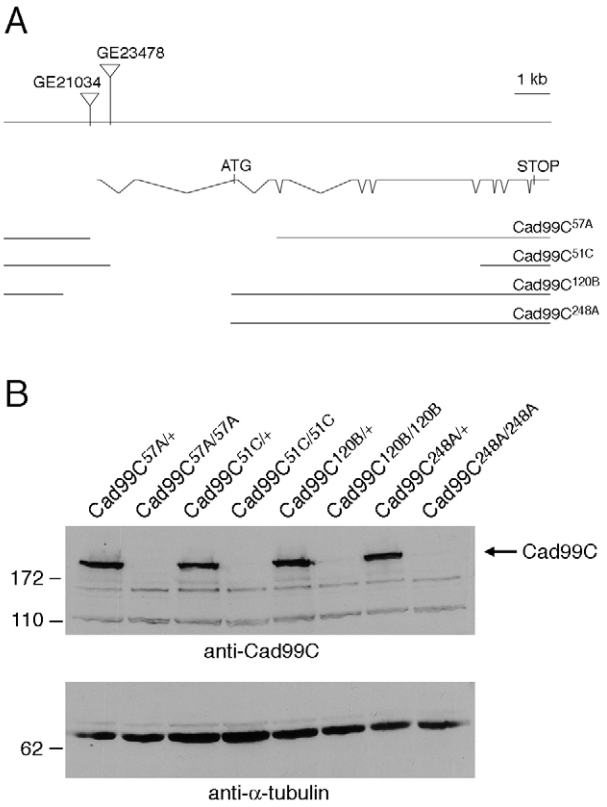


Fig. 1. Cad99C protein is not detected in ovaries of *Cad99C* mutant flies. (A) Extent of the deletions in *Cad99C* mutant alleles. The positions of the EP elements GE21034 and GE23478, which were used to generate the *Cad99C* mutant alleles, and the exon-intron structure of the *Cad99C* gene are indicated. The allele $Cad99C^{248A}$ also removes the two predicted genes CG15510 and CG31033 to the left of *Cad99C*. (B) Western blot analysis of ovaries with an anti-Cad99C antiserum. Ovaries were isolated from heterozygous $Cad99C^{57A/+}$, $Cad99C^{51C/+}$, $Cad99C^{120B/+}$, and $Cad99C^{248A/+}$ (lanes 1, 3, 5, 7) and homozygous mutant $Cad99C^{57A/57A}$, $Cad99C^{51C/51C}$, $Cad99C^{120B/120B}$, and $Cad99C^{248A/248A}$ (lanes 2, 4, 6, 8) flies. The anti-Cad99C antiserum detects a protein of 184 kDa (arrow), the expected molecular weight of Cad99C, in extracts of heterozygous, but not homozygous, *Cad99C* mutant ovaries. Subsequent blotting with an anti-α-tubulin antibody (lower panel) shows the loading of similar amounts of protein in each lane. Molecular weights are indicated to the left (in kDa).

flies took up Neutral Red (Fig. 2A,F). By contrast, more than 75% of eggs laid by homozygous mutant *Cad99C* flies took up Neutral Red (Fig. 2B-F). A similar phenotype was obtained with trans-heterozygous *Cad99C* allelic combinations inter se (Fig. 2F), indicating that the altered permeability to Neutral Red is due to the lesion in *Cad99C*.

Cad99C is required for formation of the vitelline membrane

To test whether the altered permeability to Neutral Red correlates with defects in the vitelline membrane, we fluorescently stained ultrathin longitudinal sections of stage-13 egg chambers with antibodies against the vitelline membrane proteins sV17 and sV23. Control egg chambers displayed a continuous line of sV17 and sV23 staining (Fig. 3A, and data

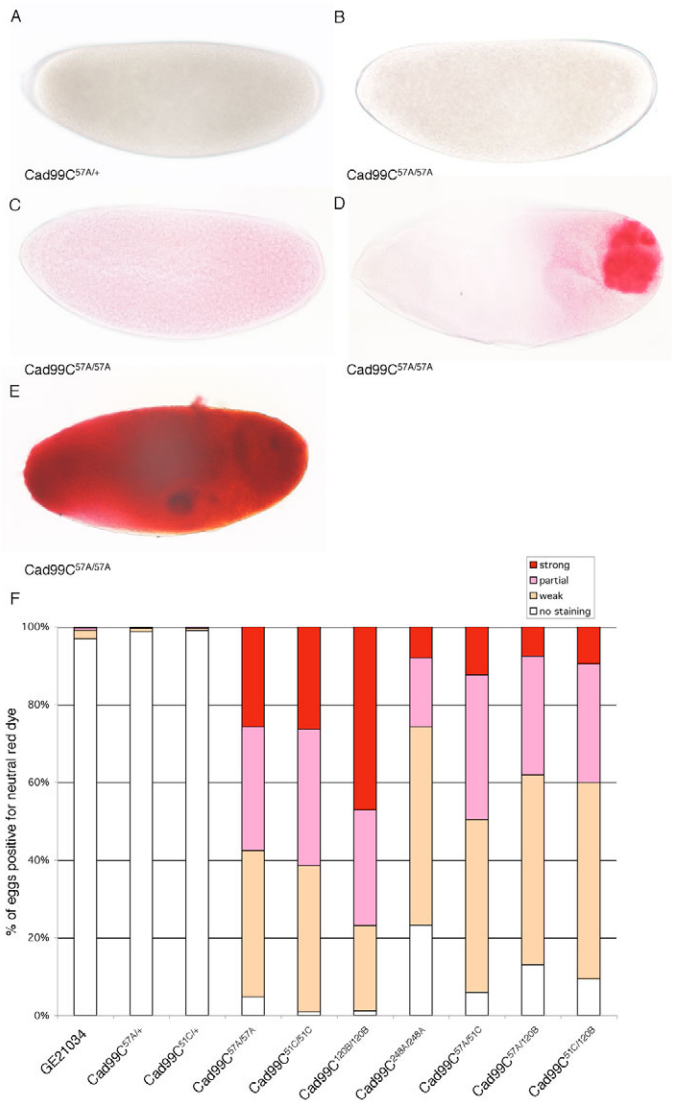


Fig. 2. Eggs laid by *Cad99C* mutant flies are permeable to the dye Neutral Red. (A-E) Eggs derived from (A) heterozygous $Cad99C^{57A/+}$ or (B-E) homozygous mutant $Cad99C^{57A/57A}$ flies were dechorionated and incubated in the dye Neutral Red. Eggs derived from homozygous mutant flies showed a range of Neutral Red stainings from (B) undetectable to (C) uniform weak, (D) partially strong and (E) uniformly strong. (F) Quantification of the percentage of eggs with no, weak, partial and strong Neutral Red staining. The genotypes of the flies from which the eggs were derived are indicated. GE21034 is the parental fly line used to generate the $Cad99C^{57A}$ and $Cad99C^{248A}$ mutants. At least 750 eggs per genotype were analyzed.

not shown). By contrast, the sV17 and sV23 stainings were disrupted in $Cad99C^{57A/57A}$ mutant egg chambers (Fig. 3B, and data not shown), suggesting that *Cad99C* is required for the formation of a continuous vitelline membrane. Stainings for the chorion proteins S18 and S36 showed continuous lines, indicating that the chorion was normal in $Cad99C^{57A/57A}$ mutant egg chambers (data not shown). To study the vitelline membrane defects further, we analyzed the vitelline membrane of egg chambers by electron microscopy. Wild-type egg chambers had a continuous vitelline membrane of even thickness (Fig. 3C). By contrast, the vitelline membrane of egg chambers from $Cad99C^{57A/57A}$ (Fig. 3D), $Cad99C^{51C/51C}$ and $Cad99C^{57A/51C}$ mutants (Fig. S1, supplementary material, and data not shown) were of uneven thickness and occasionally displayed holes. The holes in the vitelline membrane could explain the abnormal permeability of mutant eggs to the dye Neutral Red and the collapse of embryos through desiccation. Therefore, we conclude that Cad99C is required for the assembly of a proper vitelline membrane and is important for the production of normal eggs.

Cad99C is expressed in follicle cells surrounding the oocyte

To begin to understand how Cad99C might contribute to the formation of a proper vitelline membrane, we first determined in which cells of the ovary *Cad99C* RNA and protein were present. RNA in situ hybridization using a *Cad99C*-specific antisense RNA probe was performed on wild-type ovaries. Little hybridization signal was detected in the germarium and stage-2 and stage-3 egg chambers (Fig. 4A). During stages 4-8, a hybridization signal was present in the follicle cells located at the anterior and posterior poles of the egg chamber. During stages 9-14, a hybridization signal was detected in follicle cells surrounding the oocyte (Fig. 4A,B, and data not shown). No signal above background was detected in nurse cells or the stretched follicle cells surrounding the nurse cells, and only background signal was detected from a control sense *Cad99C* RNA probe (data not shown).

To determine the distribution of Cad99C protein in wild-type ovaries, we used an anti-Cad99C antiserum. Little immunoreactivity was detected in the germarium and stage-2 and stage-3 egg chambers (Fig. 4C,C'). During stages 4-8, the anti-Cad99C antiserum stained the border between the follicle cells and the oocyte at the anterior and posterior poles of the egg chamber (Fig. 4C,C'). During stages 9-14, the anti-Cad99C antiserum immunoreacted with structures at the entire border between the oocyte and the follicle cells (Fig. 4D,D', and data not shown). Follicle cells covering the nurse cells, or the nurse cells themselves, were not stained. Little immunoreactivity was detected in homozygous mutant $Cad99C^{57A/57A}$ egg chambers (Fig. 4E,E'). The detection of Cad99C protein at the border between the oocyte and the follicle cells, along with the detection of *Cad99C* RNA only in follicle cells, indicates that Cad99C protein is most probably present only in the follicle cells but not in the oocyte. Thus, both *Cad99C* RNA and protein are present at the proper time and place for Cad99C to play a role in deposition of the vitelline membrane.

Cad99C localizes to microvilli of follicle cells

The images shown in Fig. 4 indicate that Cad99C protein localizes to the border between the oocyte and surrounding follicle cells. To determine more precisely the subcellular localization of Cad99C, we stained stage-10 egg chambers with a marker for the zonula adherens, DE-cadherin, and Cad99C. Cad99C immunoreactivity was found apical to DE-cadherin (Fig. 5A-C), indicating that, unlike DE-cadherin, Cad99C does not localize to the zonula adherens, but rather to the apical plasma membrane. To test whether Cad99C protein localizes to the apical plasma membrane of follicle cells, we used the follicle-cell-specific Gal4 line CY2 in conjunction with *UAS-mCD8-GFP* to express CD8-GFP, a transmembrane

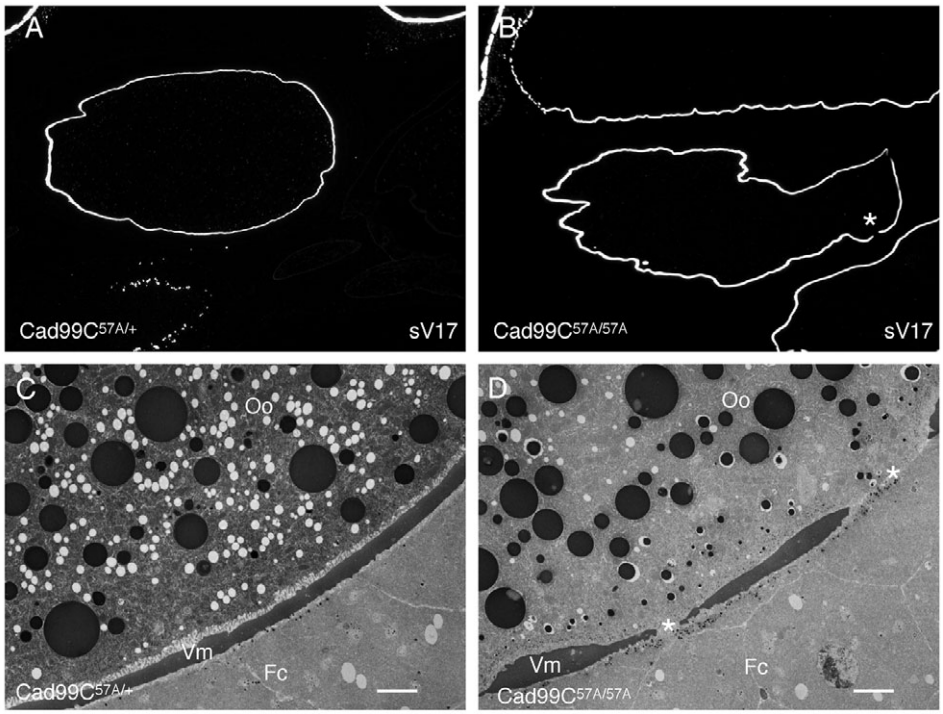
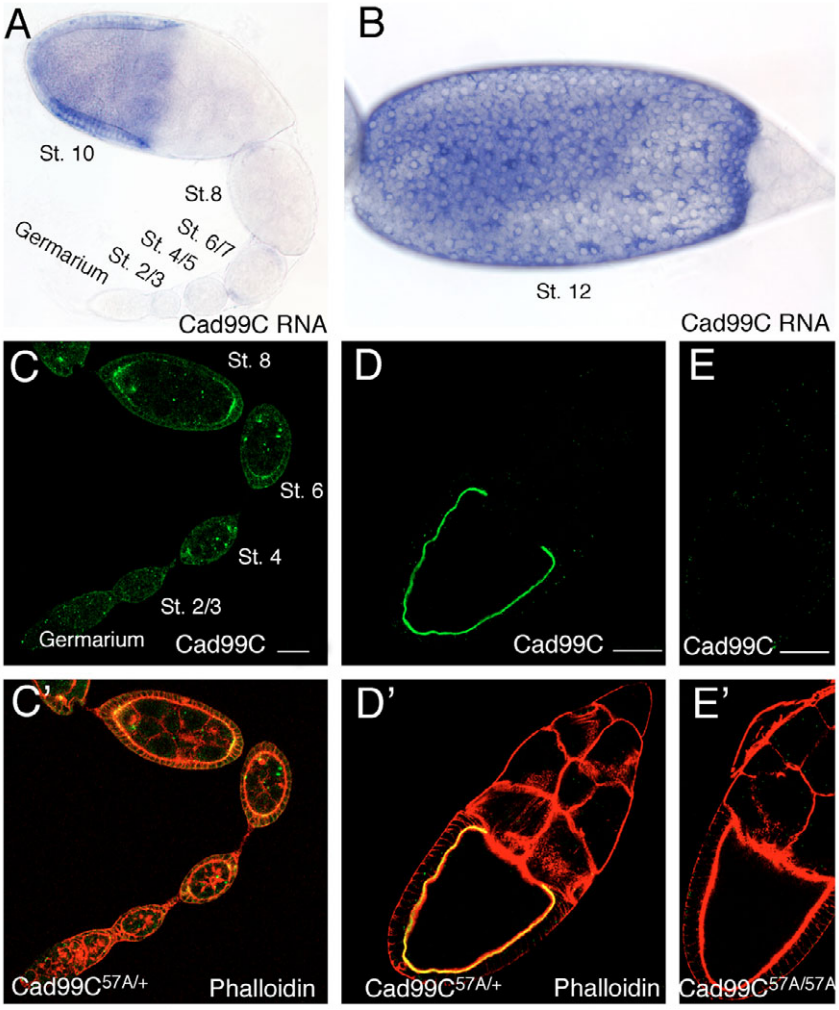


Fig. 3. The vitelline membrane of *Cad99C* mutant eggshells is unevenly deposited and displays holes. (A,B) Ultrathin sections of stage-13 egg chambers of (A) heterozygous *Cad99C*$^{57A/+}$ or (B) homozygous mutant *Cad99C*$^{57A/57A}$ flies stained for the vitelline membrane protein sV17. Control egg chambers display a continuous line of sV17 staining, whereas mutant egg chambers display sites where the sV17 staining is not detected (asterisk). (C,D) Electron micrographs of sections through stage-11 egg chambers of (C) heterozygous *Cad99C*$^{57A/+}$ or (D) homozygous mutant *Cad99C*$^{57A/57A}$ flies. The vitelline membrane (Vm) is unevenly deposited in the mutant egg chambers and is not detected at some sites (asterisks). Oo, oocyte; Fc, follicle cells. Bars, 2 μm (C,D).



protein routinely used to mark plasma membranes, in follicle cells. CD8-GFP labeled the basolateral plasma membrane as well as the apical plasma membrane. On the apical surface of follicle cells, CD8-GFP labeled protrusions that presumably represent single microvilli, or bundles containing a few microvilli (Fig. 5D). Cad99C colocalized with the apical protrusions labeled with CD8-GFP (Fig. 5E,F), consistent with the notion that Cad99C localizes to microvilli. To test whether Cad99C localizes to microvilli at the ultrastructural level, immunogold electron microscopy using an anti-Cad99C antiserum was performed on control and *Cad99C*$^{57A/57A}$ egg chambers. Very little immunoreactivity was detected on the apical plasma membrane of *Cad99C*$^{57A/57A}$ mutant egg chambers (Fig. 5H). By contrast, immunoreactivity for Cad99C was observed in control *Cad99C*$^{57A/+}$ stage-9 to stage-14 egg chambers on microvilli of follicle cells (Fig. 5G, and data not shown). At stage 10, 97% of the immunogold particles on the apical plasma membrane were present on microvilli,

Fig. 4. Cad99C is expressed in follicle cells surrounding the oocyte. (A,B) Wild-type ovaries hybridized with a *Cad99C*-specific RNA probe. (C-E') Ovaries of (C-D') heterozygous *Cad99C*$^{57A/+}$ or (E,E') homozygous mutant *Cad99C*$^{57A/57A}$ flies stained with an anti-Cad99C antiserum (green) and Rhodamine-Phalloidin (red). In (C,D,E) only the Cad99C staining is shown. Bars, 20 μm (C); 50 μm (D-E). St., stage.

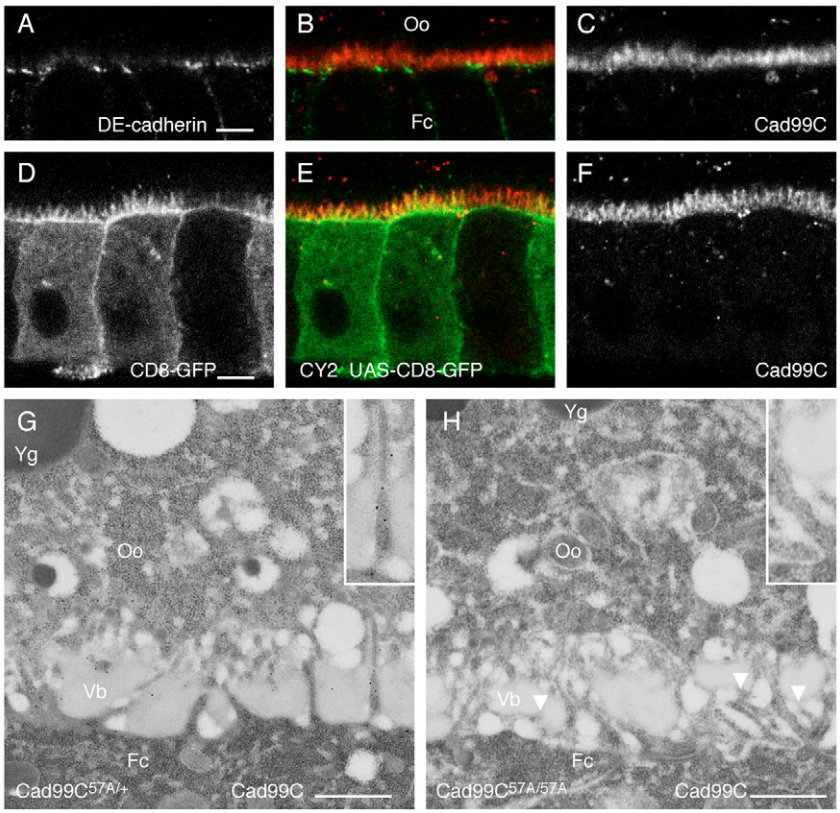


Fig. 5. Cad99C protein localizes to microvilli of follicle cells. (A-C) Follicle cells of a stage-10 wild-type egg chamber stained for (A) DE-cadherin and (C) Cad99C. (B) Merge of (A) and (C). DE-cadherin is shown in green and Cad99C in red. Apical is to the top. (D-F) Follicle cells of a stage-10 egg chamber expressing the membrane marker CD8-GFP (CY2, *UAS-CD8-GFP*) stained for (D) GFP and (F) Cad99C. (E) Merge of (D) and (F). Cad99C colocalizes with CD8-GFP to apical protrusions. Apical is to the top. (G,H) Electron micrographs of anti-Cad99C immunogold-labeled sections of stage-10 egg chambers of (G) heterozygous $Cad99C^{57A/+}$ or (H) homozygous mutant $Cad99C^{57A/57A}$ flies. Insets show higher magnification views. 10 nm immunogold particles are detected on microvilli of heterozygous $Cad99C^{57A/+}$ follicle cells, but not homozygous mutant $Cad99C^{57A/57A}$ follicle cells. Note that, in homozygous mutant $Cad99C^{57A/57A}$ flies, microvilli (arrowheads) are observed more frequently between the vitelline bodies and the planar follicle cell surface compared with control flies. Oo, oocyte; Fc, follicle cells; Yg, Yolk granule; Vb, Vitelline body. Bars, 5 μm (A-F); 500 nm (G,H).

whereas 3% were detected on the apical plasma membrane outside microvilli (n=70), indicating that Cad99C is highly enriched on the microvilli of follicle cells. Cad99C was not detected on the oocyte microvilli (Fig. 5G). Thus, Cad99C specifically localizes to microvilli of follicle cells.

Cad99C is required for normal microvilli morphology

The presence of Cad99C on follicle cell microvilli suggested to us that Cad99C might play a role in microvilli morphogenesis. To test this, we used three approaches. First, we expressed CD8-GFP as a marker for microvilli in follicle cells of control and *Cad99C* mutant flies and analyzed the morphology of CD8-GFP-labeled protrusions. In confocal microscope sections of control follicle cells, regularly spaced CD8-GFP-labeled protrusions of similar apparent length were detected (Fig. 6A). A few follicle cells of $Cad99C^{57A/51C}$ or $Cad99C^{57A/120B}$ mutant flies displayed some CD8-GFP-labeled protrusions of similar apparent length as control follicle cells, but no regularly spaced assembly of similar length protrusions was detected (Fig. 6B,C). Most *Cad99C* mutant follicle cells had CD8-GFP-labeled protrusions that appeared very short in confocal sections (Fig. 6B,C). Apart from the abnormal microvilli, the overall morphology of the mutant follicle cells was not overtly altered.

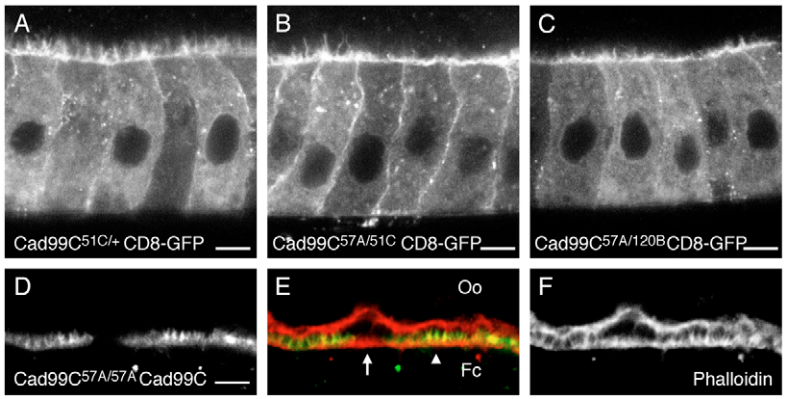


Fig. 6. Follicle cell microvilli are abnormal in *Cad99C* mutants. (A-C) Follicle cells of stage-10 (A) heterozygous $Cad99C^{51C/+}$, (B) mutant $Cad99C^{57A/51C}$, or (C) mutant $Cad99C^{57A/120B}$ egg chambers expressing CD8-GFP (CY2, *UAS-CD8-GFP*) stained for GFP. *Cad99C* mutant flies have abnormal CD8-GFP-labeled protrusions compared with control flies. Apical is to the top. (D-F) Clones of homozygous mutant $Cad99C^{57A/57A}$ follicle cells of stage-10 egg chambers stained with Rhodamine-Phalloidin marked by the absence of Cad99C. Cad99C staining is shown in (D) and Rhodamine-Phalloidin staining in (F). (E) Merge of (D) and (F). Phalloidin staining detects microvilli in between control follicle cells and the oocyte (arrowhead), but not between mutant follicle cells and the oocyte (arrow). Note that the distance between the oocyte and the $Cad99C^{57A/57A}$ follicle cells is increased. Oo, oocyte; Fc, Follicle cells. Bars, 5 μm (A-D).

Microvilli contain bundles of actin filaments at their core and can be detected by Phalloidin staining (e.g. Jimenez et al., 2002). Thus, as a second approach, we generated marked clones of follicle cells homozygous mutant for $Cad99C^{57A/57A}$ using the FRT-FLP system and analyzed the microvilli by

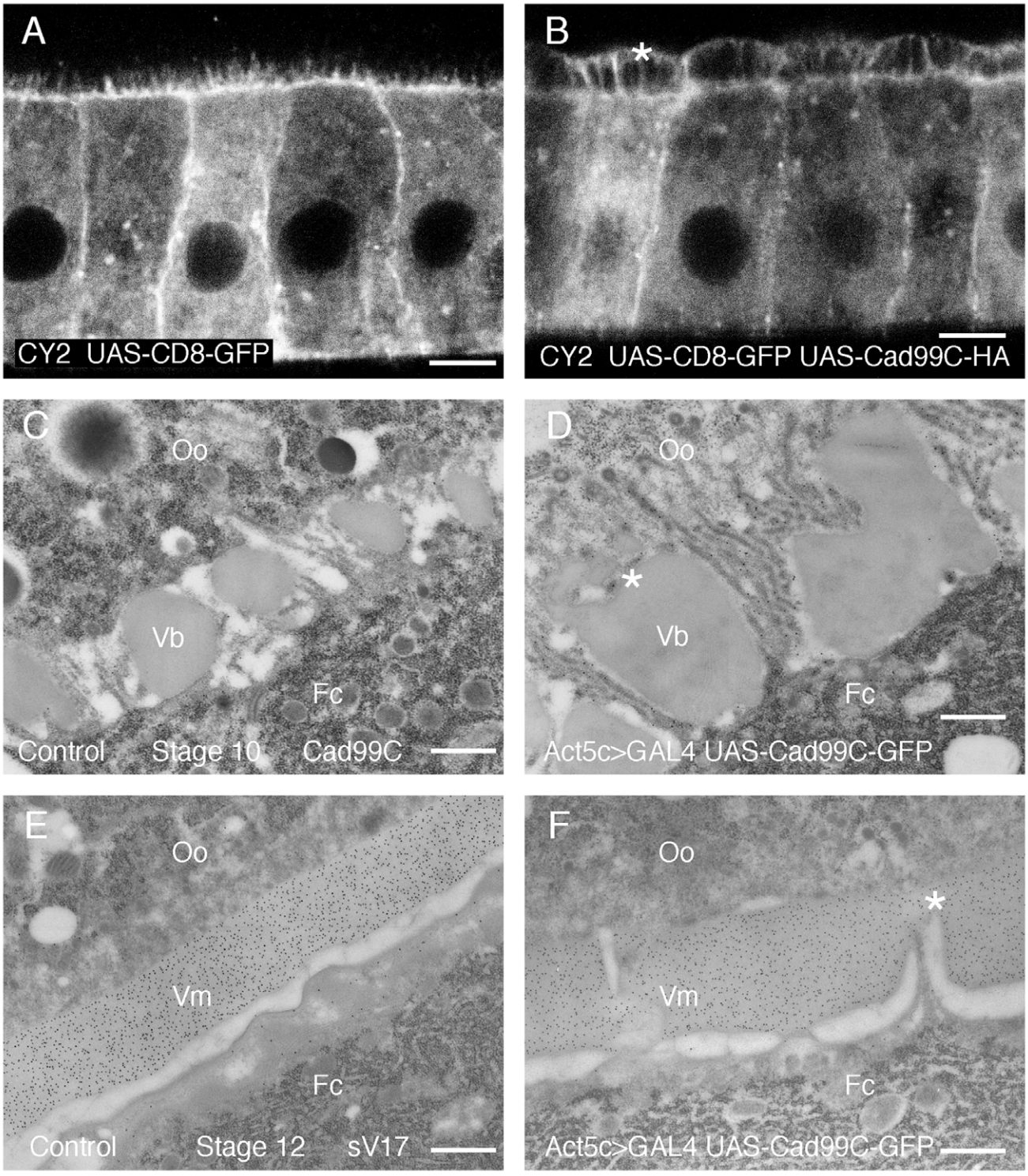


Fig. 7. Overexpression of Cad99C leads to large bundles of microvilli and vitelline membrane defects. (A,B) Follicle cells of stage-10 egg chambers expressing (A) CD8-GFP (CY2, *UAS-CD8-GFP*) or (B) CD8-GFP and Cad99C-HA (CY2, *UAS-CD8-GFP*, *UAS-Cad99C-HA*) stained for GFP. The CD8-GFP-labeled protrusions in follicle cells expressing Cad99C-HA form roof-like structures (asterisk). Apical is to the top. (C,D) Electron micrographs of anti-Cad99C immunogold-labeled sections of stage-10 (C) control egg chambers and (D) egg chambers expressing Cad99C-GFP (*Act5C>GAL4*, *UAS-Cad99C-GFP*) in clones of follicle cells. Overexpression of Cad99C-GFP results in large Cad99C-labeled bundles of follicle cell microvilli located in between vitelline bodies (Vb). Cad99C-labeled microvilli that are parallel and close to the oocyte surface are marked by an asterisk. (E,F) Electron micrographs of anti-sV17 10 nm immunogold-labeled sections of stage-12 (E) control egg chambers and (F) egg chambers expressing Cad99C-GFP (*Act5C>GAL4*, *UAS-Cad99C-GFP*) in clones of follicle cells. Long microvilli persist in follicle cells expressing Cad99C-GFP and the vitelline membrane (Vm) is disrupted (asterisk). Oo, oocyte; Fc, follicle cells. Bars, 5 μm (A,B); 500 nm (C-F).

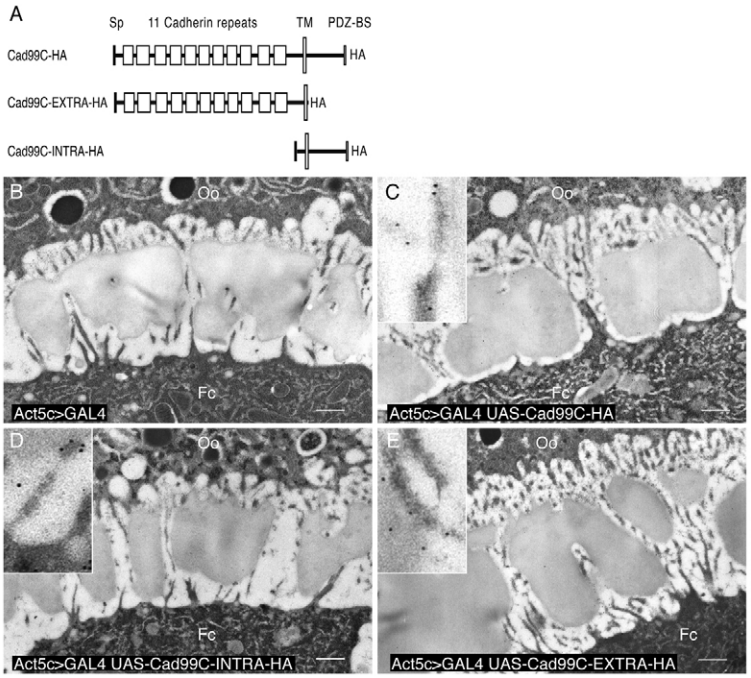


Fig. 8. The extracellular region fused to the transmembrane domain of Cad99C is sufficient to promote the formation of large bundles of follicle cell microvilli. (A) Scheme of Cad99C-HA, Cad99C-EXTRA-HA and Cad99C-INTRA-HA. Cad99C-EXTRA-HA lacks the entire cytoplasmic region, whereas Cad99C-INTRA-HA lacks most of the extracellular region. Full-length and deletion mutants of Cad99C all have a triple HA tag at their C-terminus. Sp, signal peptide; TM, transmembrane domain; PDZ-BS, putative PDZ domain-binding site. (B-E) Electron micrographs of anti-HA 12 nm immunogold-labeled sections of stage-10 (B) control egg chambers (*Act5C>GAL4*) and egg chambers expressing (C) Cad99C-HA (*Act5C>GAL4*, *UAS-Cad99C-HA*), (D) Cad99C-INTRA-HA (*Act5C>GAL4, UAS-Cad99C-INTRA-HA*), and (E) Cad99C-EXTRA-HA (*Act5C>GAL4*, *UAS-Cad99C-EXTRA-HA*) in clones of follicle cells. (C-E) Insets show higher magnification views. Overexpression of Cad99C-HA or Cad99C-EXTRA-HA results in the formation of large HA-labeled bundles of follicle cell microvilli. Oo, oocyte; Fc, follicle cells. Bars, 500 nm (B-E).

Phalloidin staining. Regular-spaced stripes of Phalloidin staining between the follicle cells and the oocyte, presumably representing microvilli, were present in the region of control *Cad99C*$^{57A/+}$ follicle cells (Fig. 6D-F). In contrast, no regular-spaced stripes of Phalloidin staining were detected in between the oocyte and homozygous mutant *Cad99C*$^{57A/57A}$ follicle cells (Fig. 6D-F).

Finally, we compared the morphology of follicle cell microvilli from control *Cad99C*$^{57A/+}$ and *Cad99C*$^{57A/57A}$ mutant stage-10 egg chambers at the ultrastructural level by electron microscopy. In the control, follicle cell microvilli were predominantly oriented towards the oocyte and were separated from one another by vitelline bodies (Fig. 5G). In contrast, microvilli of *Cad99C*$^{57A/57A}$ mutant follicle cells were mainly oriented towards neighboring follicle cells and were often observed in the space between the vitelline bodies and the apical follicle cell surface (Fig. 5H). Taken together, these data indicate that Cad99C is required for the normal morphology and organization of follicle cell microvilli.

Overexpression of Cad99C results in abnormal bundles of microvilli and defects in the vitelline membrane

As loss of function of Cad99C resulted in abnormal follicle cell microvilli, we next tested whether overexpression of Cad99C might also alter microvillar morphology. To this end, we co-expressed a HA-tagged version of Cad99C with CD8-GFP in follicle cells using the Gal4 line CY2. Compared with control follicle cells expressing only CD8-GFP, the follicle cells co-expressing Cad99C-HA and CD8-GFP had more prominent CD8-GFP-labeled protrusions that formed roof-like structures (Fig. 7A,B). Similar results were obtained by co-expressing Cad99C-HA and CD8-GFP in clones of follicle cells using the *Act5C>GAL4* driver line (data not shown).

To examine the microvilli in Cad99C-overexpressing cells at an ultrastructural level, we analyzed clones of follicle cells expressing a GFP-tagged Cad99C, Cad99C-GFP, under the control of the *Act5C>GAL4* driver line by immunoelectron microscopy using an anti-Cad99C antiserum. Electron micrographs of Cad99C-GFP-expressing follicle cells showed large bundles of Cad99C-labeled, parallel, sectioned microvilli between the vitelline bodies that were not observed in electron micrographs of control follicle cells (Fig. 7C,D). In addition, sectioned Cad99C-labeled microvilli were detected close, and aligned parallel, to the surface of the oocyte (Fig. 7D). The latter microvilli might reflect the CD8-GFP-labeled roof-like structures that were observed when Cad99C-HA and CD8-GFP were co-expressed.

During stages 11-12 of oogenesis, the microvilli of wild-type follicle cells shorten and the vitelline bodies coalesce into a continous vitelline membrane (Mahowald, 1972; Mahowald and Kambysellis, 1980) (Fig. 7E). However, when Cad99C-GFP was expressed in follicle cells, some inappropriately long microvilli persisted through stage 12, interrupting the vitelline membrane (Fig. 7F), indicating that overexpression of Cad99C prevents the timely shortening of microvilli. Together, these data indicate that overexpression of Cad99C is sufficient to alter the morphology of follicle cell microvilli, resulting in large bundles of microvilli and defects in the vitelline membrane.

The extracellular region of Cad99C promotes the formation of large microvilli bundles

The above experiments indicated that expression of Cad99C promotes the formation of abnormally large bundles of follicle cell microvilli. To gain insights into the mechanisms used by Cad99C to induce large bundles of microvilli, we undertook a structure-function analysis. Cad99C contains extracellular domains, such as cadherin repeats, as well as intracellular motifs, such as a PDZ-binding site, that could be important for promoting the bundling of follicle cell microvilli, for example, through interactions with other proteins. To address whether the extracellular region, the intracellular region, or both regions

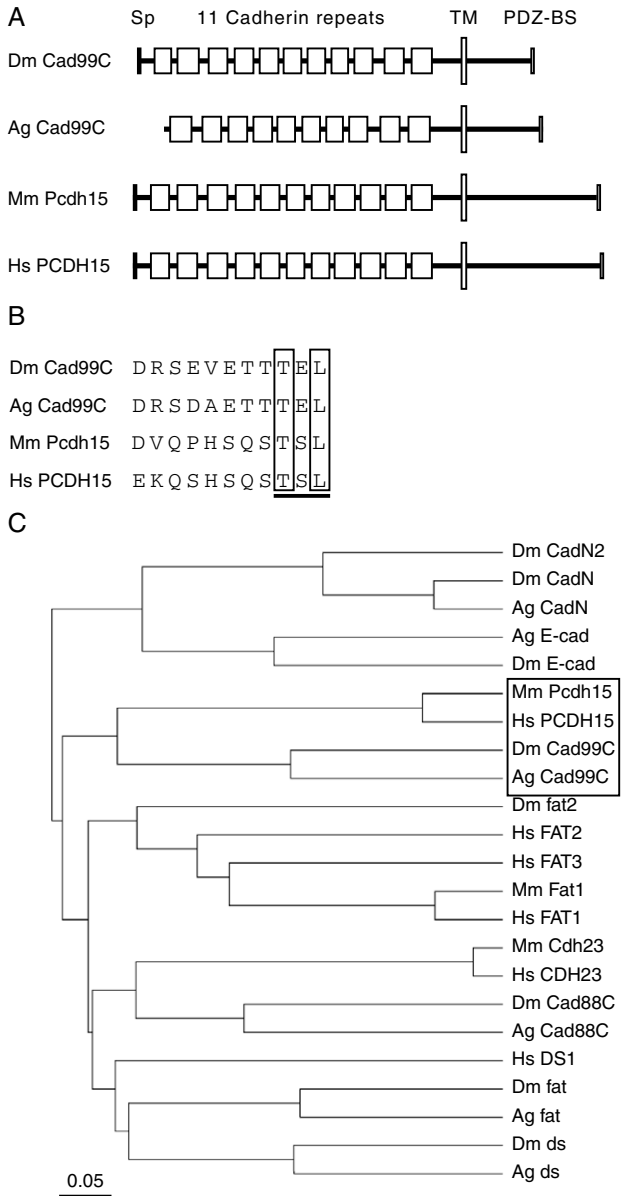


Fig. 9. *Drosophila* Cad99C is closely related to human PCDH15. (A) *Drosophila melanogaster* (Dm) Cad99C, *Anopheles gambiae* (Ag) Cad99C, *Mus musculus* (Mm) Pcdh15 and *Homo sapiens* (Hs) PCDH15 share a similar protein domain organization. They are single-span transmembrane (TM) proteins containing a signal peptide (Sp) and 11 cadherin repeats in their extracellular regions. The cytoplasmic region has a conserved PDZ domain-binding site (PDZ-BS). The available *Anopheles* Cad99C sequence is incomplete at its N-terminus. (B) Conservation of a C-terminal PDZ domain-binding site between the four proteins. The cytoplasmic regions of *Drosophila* and *Anopheles* Cad99C, and mouse Pcdh15 and human PCDH15 contain conserved C-terminal class I PDZ domain-binding sites (underlined) with the consensus sequence (S/T)XL (Sheng and Sala, 2001). (C) Phylogenetic tree built with the UPGMA method. Dm *Cad99C*, Ag *Cad99C*, Mm *Pcdh15* and Hs *PCDH15* segregate into the same clade (box), indicating that *Cad99C* and *PCDH15* share a common evolutionary history and are more related to each other than to any other of the cadherins considered. The non-classical cadherins Dm and Ag *Cad88C* appear to be related to Hs *cadherin 23* (*CDH23*) and Mm *Cdh23*; like *PCDH15*, *CDH23* is etiologically associated with Usher syndrome type I.

of Cad99C were required to promote the formation of large microvilli bundles, we generated mutant versions of Cad99C lacking either the intracellular or the extracellular region, Cad99C-EXTRA-HA and Cad99C-INTRA-HA, respectively (Fig. 8A, and Materials and Methods). Clones of follicle cells expressing full-length Cad99C-HA, Cad99C-EXTRA-HA and Cad99C-INTRA-HA were analyzed by immunoelectron microscopy using an anti-HA antibody. Anti-HA immunoreactivity for all three proteins was detected on follicle cell microvilli (Fig. 8C-E). Follicle cells expressing Cad99C-EXTRA-HA showed large bundles of HA-labeled, parallel, sectioned microvilli similar to those observed when full-length Cad99C-HA was expressed (Fig. 8C,E). Expression of Cad99C-INTRA did not result in large bundles of microvilli (Fig. 8D). Thus, the extracellular region of Cad99C fused to the transmembrane domain of Cad99C is sufficient to promote the formation of microvilli bundles, suggesting that the extracellular region of Cad99C is important for the function of Cad99C in organizing follicle cell microvilli.

Drosophila Cad99C is closely related to human protocadherin 15

Microvilli are common to epithelial cells in most organisms. We therefore tested whether protein sequences similar to Cad99C were also present in other organisms. The entire *Drosophila* Cad99C protein sequence was used to search databases for Cad99C-related proteins using the BLAST algorithm. In addition to a putative Cad99C *Anopheles gambiae* homolog (XP_312660; E-value: 0.0), BLAST also identified protocadherin 15 proteins from *Mus musculus* (AAG53891; E-value: e-105), *Homo sapiens* (AAK31804, E-value: e-104), *Tetraodon nigroviridis* (CAG12628, E value: e-100) and other species as putative Cad99C homologs. Human PCDH15 is a non-classical member of the cadherin superfamily that localizes to stereocilia, microvilli-derived extensions of inner ear cells (Ahmed et al., 2003). Mutations in *PCDH15* are etiologically associated with non-syndromic deafness and Usher syndrome type 1F, an autosomal recessive disease characterized by hearing loss, vestibular dysfunction and retinopathy (Ahmed et al., 2001; Alagramam et al., 2001b). A back-BLAST analysis using human PCDH15 as the query sequence identified *Drosophila* Cad99C as the sequence from *Drosophila* with the highest similarity (E-value: 4e-98) to human PCDH15, suggesting that Cad99C is the cadherin in *Drosophila* that is most closely related to human PCDH15, and vice versa.

To analyze further the relationship between Cad99C and PCDH15, we compared the domain organization of *Drosophila* and *Anopheles* Cad99C with that of the human and mouse proteins. The four proteins share a similar structural organization, with an extracellular region containing 11 cadherin repeats (however, the available *Anopheles* Cad99C sequence is N-terminally truncated and has only 10 cadherin repeats), a single-span transmembrane domain, and a cytoplasmic region with a conserved C-terminal class I PDZ domain-binding site (Fig. 9A,B). Cad99C is the only one among the 17 cadherin-like sequences in *Drosophila* that has 11 cadherin repeats (Hill et al., 2001; Hynes and Zhao, 2000).

To estimate the evolutionary relationship between *Cad99C* and *PCDH15*, we built a phylogenetic tree based on the alignment of the full-length sequences of the cadherins most

closely related to Cad99C (see Materials and Methods). *Drosophila* and *Anopheles Cad99C* and mouse *Pcdh15* and human *PCDH15* segregated into the same clade (Fig. 9C), suggesting that Cad99C and PCDH15 are closely related. Thus, based on BLAST analysis, the conserved protein domain organization and our phylogenetic analysis, we suggest that Cad99C and PCDH15 are homologs.

Discussion

In this study, we provide several lines of evidence suggesting a role for the cadherin Cad99C in establishing or maintaining the morphology of the microvilli present on the apical surface of follicle cells surrounding the oocyte in *Drosophila*. First, *Cad99C* RNA and protein are present in follicle cells surrounding the oocyte at a time when these cells display long microvilli. Second, Cad99C protein localizes to follicle cell microvilli. Third, mutations in *Cad99C* lead to disorganized and abnormal microvilli. Fourth, overexpression of Cad99C results in large bundles of microvilli. This study, as well as a recently published study (D'Alterio et al., 2005), thus reveals a novel role for a cadherin family member during *Drosophila* development.

Cad99C localizes to follicle cell microvilli

Follicle cells surrounding the oocyte display microvilli whose lengths are developmentally regulated. The onset of Cad99C expression in most follicle cells correlates with the appearance of long microvilli. At stage 10, when long microvilli are most prominent, we demonstrate by immunoelectron microscopy that Cad99C localizes to, and is enriched on, follicle cell microvilli.

Localization to microvilli is not typical of many cadherins, and Cad99C is so far the only cadherin in *Drosophila* that has been localized to microvilli. It is noteworthy that, even in vertebrates, only a few cadherins have been localized to microvilli or microvilli-derived cellular extensions. Among them are PCDH15 and cadherin 23 (CDH23), two cadherins that localize to stereocilia, which are microvilli-derived extensions of cochlear hair cells important for auditory perception in vertebrates (Ahmed et al., 2003; Boeda et al., 2002; Lagziel et al., 2005; Michel et al., 2005; Rzadzinska et al., 2005; Siemens et al., 2004). Both *PCDH15* and *CDH23* are etiologically associated with non-syndromic deafness and Usher syndrome type 1, a disease characterized by hearing loss, vestibular dysfunction and retinopathy (Ahmed et al., 2001; Alagramam et al., 2001b; Bolz et al., 2001; Bork et al., 2001; Di Palma et al., 2001). Stereocilia normally display an ordered, staircase-like arrangement on the surface of hair cells and are held together by several molecular links (reviewed by Frolenkov et al., 2004). In the absence of PCDH15, stereocilia do not display their normal ordered arrangement, and are instead splayed (Alagramam et al., 2001a; Seiler et al., 2005). On the basis of its localization and mutant phenotype, it has been proposed that PCDH15 bundles stereocilia by providing adhesive links between their lateral membranes (Ahmed et al., 2003). We find, using bioinformatics, that Cad99C is closely related to PCDH15, indicating that insects and vertebrates have employed related cadherins to organize some microvilli-like cellular extensions. Our results also indicate that the organization of cellular extensions might be a more general function of cadherins not restricted to stereocilia.

Cad99C is required for the normal morphology of follicle cell microvilli

We present several lines of evidence indicating that Cad99C is required for establishing or maintaining the normal morphology of follicle cell microvilli. First, regular-spaced CD8-GFP-labeled protrusions present in control follicle cells were not detected in follicle cells of *Cad99C* mutant flies. Second, in clones of follicle cells mutant for $Cad99C^{57A/57A}$, we failed to detect the prominent regular-spaced stripes of Phalloidin staining detected in between the oocyte and control $Cad99C^{57A/+}$ follicle cells. The latter result indicates that Cad99C is required for normal microvilli morphology in follicle cells, consistent with the finding that Cad99C localizes to microvilli of follicle cells rather than the oocyte. The failure to detect regular-spaced CD8-GFP-labeled protrusions or Phalloidin stripes between follicle cells and the oocyte by confocal microscopy in *Cad99C* mutant flies suggests that the morphology of microvilli is altered. For example, microvilli either may be mis-oriented so that they come to lie more parallel and closer to the surface of the follicle cells, or microvilli may be shorter. The frequent detection of microvilli located between the vitelline bodies and the planar follicle cell surface in *Cad99C* mutant flies by electron microcopy (Fig. 5H) indicates that follicle cell microvilli are mis-oriented.

The expression of Cad99C is not restricted to follicle cells but is rather widespread among epithelia, including wing imaginal discs (Schlichting et al., 2005), raising the possibility that Cad99C might also be required for normal microvilli morphology in epithelial cells other than follicle cells. We have analyzed the ultrastructure of wing imaginal disc cell microvilli and do not find evidence that Cad99C is required for maintaining their normal morphology (Fig. S2, supplementary material). Cad99C might act redundantly with another protein in the morphogenesis of wing imaginal disc cell microvilli. Alternatively, Cad99C might not play a general function in the morphogenesis of microvilli of epithelial cells but its function might instead be restricted to the microvilli of follicle cells.

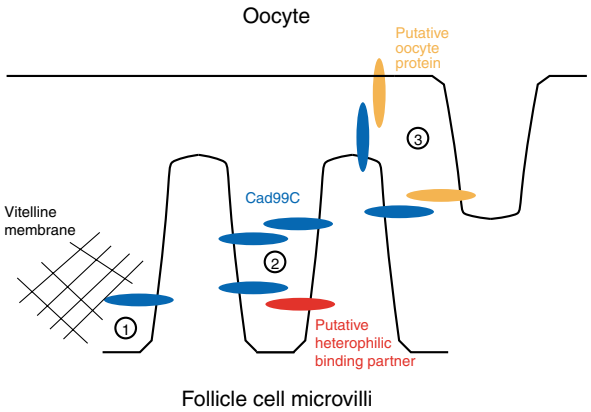


Fig. 10. Model for Cad99C function. Cad99C is present on follicle cell microvilli and, through its extracellular cadherin repeats, could establish molecular links to the extracellular matrix, to Cad99C or to a different heterophilic binding partner on a neighboring microvillus, or to a protein located on the oocyte surface. These molecular links could stabilize or promote the assembly of microvilli by providing physical adhesion of the microvilli to a target and/or signaling to the cytoskeleton.

Microvilli and the vitelline membrane

The vitelline membrane is unevenly deposited in *Cad99C* mutants. How does Cad99C present on follicle cell microvilli affect the assembly of the vitelline membrane? Follicle cell microvilli are closely apposed to vitelline bodies, consistent with the idea that microvilli might play a role in vitelline membrane assembly. The uneven deposition of the vitelline membrane in *Cad99C* mutant ovaries might therefore be a consequence of abnormal microvilli. Microvilli could play one of several potential roles during vitelline membrane assembly. First, microvilli might serve as sites of secretion for vitelline membrane components or proteins required for vitelline membrane assembly. However, secretory vesicles containing the vitelline membrane proteins sV17 and sV23 are predominantly distributed within the apical region of the follicle cells excluding the microvilli (Trougakos et al., 2001, and data not shown). Second, the microvilli of follicle cells and the oocyte are in close proximity; thus, one role of follicle cell microvilli might be that they mediate signaling between the oocyte and follicle cells important for vitelline membrane assembly. Consistent with a role of the oocyte in vitelline membrane assembly, mutations in the genes *fs(1)Nasrat* and *fs(1)polehole*, which encode proteins secreted by the oocyte, lead to vitelline membrane defects similar to those observed in *Cad99C* mutants (Degelmann et al., 1990; Jimenez et al., 2002). Finally, microvilli might provide a scaffold in which vitelline bodies form and thus could help to separate individual vitelline bodies and to constrain or maintain their sizes. In the absence of Cad99C, the disorganized and abnormal microvilli may not sufficiently physically separate individual vitelline bodies, resulting in some inappropriately sized vitelline bodies and ultimately in the formation of an uneven vitelline membrane.

Models for Cad99C function

How could Cad99C play a role in the formation or maintenance of normal follicle cell microvilli? Cadherins are known to provide molecular links between plasma cell membranes [and sometimes between cell membranes and the extracellular matrix (Senzaki et al., 1999)] through homophilic or heterophilic binding of the extracellular cadherin repeats on adjacent plasma membranes and the interaction of their intracellular region with the cytoskeleton (Gumbiner, 2005; Patel et al., 2003). These links are important for physical adhesion and/or cell-to-cell signaling (Wheelock and Johnson, 2003). Cad99C may, therefore, constitute a molecular link used to stabilize or promote the assembly of follicle cell microvilli by providing physical adhesion of the microvilli to a target and/or signaling to the cytoskeleton. Our finding, that the extracellular region of Cad99C fused to its transmembrane domain is sufficient to promote the formation of large microvilli bundles, indicates that the intracellular region of Cad99C might be dispensable for this function of Cad99C, and that Cad99C might mainly act through its extracellular region. Therefore, a direct interaction between Cad99C and the cytoskeleton may not be important. However, Cad99C may indirectly interact with the cytoskeleton, for example by binding with its extracellular region to a cytoskeleton-linked transmembrane protein.

If Cad99C does serve as a molecular link, it could establish, through its extracellular cadherin repeats, a molecular link to three different targets (Fig. 10). First, Cad99C present on follicle cell microvilli might interact with a component of the vitelline membrane. Second, Cad99C molecules present on neighboring follicle cell microvilli might interact homophilically with one another or heterophilically with a different binding partner, resulting in the crosslinking or bundling of microvilli [as shown by electron microscopic preparations of wild-type ovaries that demonstrated several follicle cell microvilli in close proximity (e.g. Fig. 7C)]. On the basis of this model, microvilli bundles would be less efficiently formed or maintained in *Cad99C* mutants, resulting in the destabilization or mis-orientation of microvilli, whereas overexpression of Cad99C would result in abnormally large bundles of microvilli. Indeed, mis-oriented follicle cell microvilli were detected in *Cad99C* mutant flies and abnormally large bundles of microvilli were detected upon overexpression of Cad99C in follicle cells. In this scenario, the role of Cad99C would be mechanistically similar to the proposed function of PCDH15 in bundling human stereocilia (Ahmed et al., 2003). Finally, follicle cell microvilli closely approach the surface of the oocyte and form gap junctions with the oocyte (Mahowald and Kambysellis, 1980). Thus, as a third potential target, Cad99C might provide a molecular link between follicle cell microvilli and the oocyte by interacting with a protein located at the oocyte plasma membrane. This interaction would most probably be heterophilic, as we have been unable to immunolocalize Cad99C to the oocyte plasma membrane. In this model, the absence of Cad99C would cause follicle cell microvilli to no longer be efficiently linked to the oocyte, leading to their destabilization and/or mis-orientation. By contrast, overexpression of Cad99C in follicle cells could further stabilize microvilli at the surface of the oocyte, resulting in the observed roof-like structures (Fig. 7B). It might be advantageous to employ heterophilic rather than homophilic interactions to link follicle cell microvilli to the oocyte as the presence of homophilic adhesion molecules on microvilli might result in an excess of bundling of microvilli. The identification of proteins interacting with Cad99C could help reveal the target(s) of Cad99C and, in addition, help further elucidate the function of Cad99C on microvilli.

Materials and Methods

Molecular cloning and *Drosophila* stocks

The *UAS-Cad99C-HA* transgene was generated by cloning a cDNA containing 93 bp of the 5′ untranslated region (splice variant A) and the entire coding sequence of *Cad99C* (Schlichting et al., 2005) 5′ to, and in frame with, a triple HA epitope encoding sequence derived from the influenza virus hemagglutinin protein HA1 (Wilson et al., 1984) and inserting the construct into the pUAST vector (Brand and Perrimon, 1993). The *UAS-Cad99C-GFP* transgene was generated by cloning a cDNA containing 93 bp of the 5′ untranslated region (splice variant A) and the entire coding sequence of *Cad99C* (Schlichting et al., 2005) 5′ to, and in frame with, the coding sequence for enhanced green fluorescent protein (EGFP; Clontech) and inserting the construct into the pUAST vector (Brand and Perrimon, 1993). The *UAS-Cad99C-EXTRA-HA* transgene was generated by amplifying by PCR the coding sequences for amino acids 1-1419 of Cad99C, comprising the extracellular and transmembrane regions, using the primers 5′-CGGGGTACCATAACCTTTGT-TAACGGCGTGTGACTCC-3′ and 5′-TTGGCGCGCAGAGATGCATATGTAAA-TGATGCCCAGG-3′ (the underlined sequences are *Kpn*I and *Bss*HII restriction sites used for cloning). The PCR product was then cloned 5′ to, and in frame with, the triple HA-epitope-encoding sequence and inserted into the pUAST vector. For generating the *UAS-Cad99C-INTRA-HA* transgene, two different PCR reactions were performed. First, the coding sequence for amino acids 1-36 of Cad99C, containing the signal peptide (amino acids 1-29) and a spacer region (amino acids 30-36), was amplified using the primers 5′-GGGGTACCTGTTAACGGCGTGTG-ACTCC-3′ and 5′-CCGGAATTCTTCC ACTTCGCACATCTGCGACTTGCCC-3′ (the underlined sequences are *Kpn*I and *Eco*RI restriction sites used for cloning).

Second, the coding sequence for amino acids 1397-1706 of Cad99C, containing the transmembrane and cytoplasmic regions and a triple HA-epitope-encoding sequence, was amplified using the primers 5′-CCGGAATTCCCCTTCACTC-TGATTGCCATATC-3′ and 5′-GCTCGAATTCAAGCTTTCAGCTAGC-3′ (the underlined sequences are *Eco*RI restriction sites used for cloning). The two PCR products were then cloned in frame and inserted into the pUAST vector. For each of the transgenes, the correct nucleotide sequences of the cloned PCR products were confirmed by sequencing prior to injection into y^1 w^{1118} embryos to obtain transgenic flies.

The following fly stocks were used: $Cad99C^{57A}$ and FRT82 $Cad99C^{57A}$ (Schlichting et al., 2005); $Cad99C^{51C}$, $Cad99C^{120B}$, $Cad99C^{248A}$ (this study), CY2 (Queenan et al., 1997); *UAS-mCD8-GFP* (Lee and Luo, 1999); *Act5C>CD2>GAL4* (Pignoni and Zipursky, 1997); GE21034 and GE23478 (GenExel). The generation of mutant *Cad99C* alleles was described previously (Schlichting et al., 2005). $Cad99C^{57A}$ and $Cad99C^{248A}$ were derived from GE21034; $Cad99C^{51C}$and $Cad99C^{120B}$ were derived from GE23478. The alleles $Cad99C^{51C}$, $Cad99C^{120B}$ and $Cad99C^{248A}$ contain genomic deletions spanning from 3572 bp 5′ to 7174 bp 3′, 4999 bp 5′ to 75 bp 5′, and 17980 bp 5′ to 156 bp 5′ of the *Cad99C* translational start codon, respectively. Marked clones of mutant cells were generated by Flp-mediated mitotic recombination (Golic and Lindquist, 1989; Xu and Rubin, 1993), subjecting flies once to a 35°C heat shock for 30 minutes (*Act5C>CD2>GAL4*) or three times to a 38°C heat shock for 30 minutes (*FRT82* $Cad99C^{57A}$).

Western blot of ovaries

Ovaries were dissected in ice-cold Ringer's solution. For each sample, ovaries from two flies were collected. Samples were processed as previously described (Schlichting et al., 2005). Primary antibodies were polyclonal rabbit anti-Cad99C, 1:1000 (Schlichting et al., 2005), and mouse anti-α-tubulin, 1:10,000 (T9026; Sigma). Secondary antibodies were goat anti-rabbit HRP conjugated, 1:10,000 (111-035-144; Jackson ImmunoResearch) and goat anti-mouse HRP-conjugated, 1:10,000 (31430; Pierce).

Neutral Red assay

The Neutral Red assay was performed as described previously (LeMosy and Hashimoto, 2000). The variability in uptake of Neutral Red has been previously observed for other mutants (e.g. LeMosy and Hashimoto, 2000) and has been attributed to differences in the age of the eggs or the efficiency of dechorionation.

RNA in situ hybridization

Ovaries were dissected in PBS and fixed for 10 minutes in a mixture of 100 μl devitellinizing buffer [0.15 mM $MgCl_2$, 25 mM NaCl, 1.5 mM Na-phosphate (pH 7.0), 6% formaldehyde] and 600 μl heptane. Dissected ovaries were then washed twice with PBT (PBS, 0.1% Tween 20) and incubated in 4 μg/ml proteinase K (Fluka) in PBT for 2 minutes. Proteinase K digestion was stopped by washing the dissected ovaries once for 1 minute with 2 mg/ml glycine. Dissected ovaries were then washed twice for 5 minutes with PBT, fixed in 4% paraformaldehyde for 20 minutes, and washed three times for 5 minutes in PBT. Hybridization was performed as described previously for imaginal discs (Schlichting et al., 2005). Dissected ovaries were then washed four times for 15 minutes in hybridization buffer (HB), two times for 15 minutes in HB:PBT (1:1) and five times for 10 minutes in PBT, each at 60°C. Detection and generation of the *Cad99C* antisense RNA probe were described previously (Schlichting et al., 2005).

Immunohistochemistry

Ovaries dissected from adult flies were fixed and stained with the following primary antibodies: polyclonal rabbit anti-Cad99C, 1:10,000 (Schlichting et al., 2005); polyclonal rabbit anti-sV17, 1:1000 (Pascucci et al., 1996); polyclonal rabbit anti-sV23, 1:10,000 (Pascucci et al., 1996); polyclonal rabbit anti-S18, 1:1500 (Pascucci et al., 1996); polyclonal rabbit anti-S36, 1:1000 (Pascucci et al., 1996); rat anti-DE-cadherin (DCAD2), 1:100 (Oda et al., 1994); and monoclonal mouse anti-GFP, 1:2000 (8362-1; Clontech). Secondary antibodies, all diluted 1:200, were goat anti-mouse Alexa Fluor 488, goat anti-rabbit Alexa Fluor 488, goat anti-rabbit Alexa Fluor 594 (Molecular Probes), and donkey anti-rat (712095153; Jackson ImmunoResearch). Rhodamine-Phalloidin (R-415; Molecular Probes) was used at a dilution of 1:200. Images were recorded on a LSM510 Zeiss confocal microscope. Immunofluorescence staining of ultrathin sections mounted on coverslips was performed as described previously (Wilsch-Bräuninger et al., 1997). Preparations were imaged using a Zeiss Axioplan microscope equipped with a CCD camera (Diagnostic Instruments).

Electron microscopy

Ovaries were embedded in Embed 812 (Science Services) for standard electron microscopy as described (Wilsch-Bräuninger et al., 1997). For immunogold labeling, the ovaries were embedded in Lowicryl HM20 (Science Services) as previously described (Wilsch-Bräuninger et al., 1997) using a Leica AFS device (Leica Microsystems). For immunolabeling, the following antibodies were used: rabbit anti-Cad99C, 1:500, pre-absorbed against ovaries from $Cad99C^{57A/57A}$ mutant flies (Schlichting et al., 2005); rabbit anti-sV17, 1:1000 (Pascucci et al., 1996); rabbit anti-S18, 1:1500 (Pascucci et al., 1996); and mouse anti-HA 12CA5, 1:1000 (Roche). Protein A coupled to 10 nm gold particles (Utrecht University, NL), or a goat anti-mouse secondary antibody coupled to 12 nm gold particles (Dianova), was used to detect the primary antibodies. Sections were viewed on a Morgagni electron microscope (FEI Company). Egg chambers were staged according to King (King, 1970).

Wing imaginal discs of late third instar larvae were embedded in Embed 812 (Science Services) for standard electron microscopy, as previously described (Wilsch-Bräuninger et al., 1997). AnalySIS Pro 3.2 (Soft Imaging System) was used to measure the length of microvilli. Microvilli present throughout the wing disc pouch were analyzed. Statistical analysis was performed using the Student's *t*-test.

Bioinformatic analysis

BLAST searches (Altschul et al., 1997) were performed against the non-redundant protein National Center for Biotechnology Information (NCBI) database. The domain organization of cadherins was predicted employing the Simple Modular Architecture Research Tool (SMART) (Schultz et al., 1998). The C-terminal regions containing the PDZ domain-binding sites were aligned with ClustalW v1.4 (included in MacVector 7.2, Accelrys). For the phylogenetic analysis, we considered only the cadherin sequences from *Mus musculus*, *Homo sapiens*, *Drosophila melanogaster* and *Anopheles gambiae* that, from the BLAST analysis, appeared to be most closely related to the Dm Cad99C sequence. The alignment of the full-length sequences was used to reconstruct an evolutionary tree with the Neighbor Joining and Unweighted Pair Group Method with Arithmatic Mean (UPGMA) methods using MacVector 7.2. The accession numbers of the sequences used for the bioinformatic analysis are: Dm Cad88C (NM_169594), Ag Cad88C (XP_321514), Dm E-cad (Q24298), Ag E-cad (XP_308578), Hs CDH23 (NM_022124), Mm Cdh23 (NM_023370), Dm CadN2 (AAF53636), Dm Ds (AAF51468), Ag Ds (XP_317578), Hs DS1 (NM_003737), Ag Fat (XP_317558), Dm Fat (AAF51036), Hs FAT1 (NP_005236), Mm Fat1 (CAB65271), Hs FAT2 (NP_001438), Dm Fat2 (AAF49078), Hs FAT3 (XP_061871), Dm CadN (O15943), Ag CadN (XP_318803), Mm Pcdh15 (NP_075604), Hs PCDH15 (AAK31804), Ag Cad99C (XP_312660) and Dm Cad99C (AAF56955).

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