Identification and Characterization of Interaction Partners of *Drosophila* Cadherin 99C

by

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A thesis submitted in conformity with the requirements for the degree of Master's of Science
Department of Cell and Systems Biology
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Abstract

Drosophila cadherin Cad99C is a critical regulator of follicle cell microvilli during Drosophila oogenesis. I extended the functional analysis of Cad99C by identifying Drosophila Myosin VIIA, and Cad74A and Cad87A, which are homologues of human Myosin VIIA and Cadherin 23, respectively, as components of the Cad99C protein complex and by studying their interactions in follicle cell microvilli. My co-immunoprecipitation data show that the cytoplasmic tail of Cad99C interacts with Myosin VIIA, and the extracellular cadherin repeats of Cad99C interact with Cad87A and Cad74A, independent of the cytoplasmic tail of Cad99C. Genetic studies indicate that 1) Cad99C and Cad74A/Cad87A localize independently to microvilli, although their amount is affected by changes in the ratio of their concentrations. 2) All cadherins affect microvillus morphology when overexpressed although the phenotypes are different. The overexpression effect of Cad74A depends on Cad99C but not vice versa. These data point to complex interactions between the microvillus cadherins.
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Chapter 1
Introduction

Microvilli are finger-like extensions of the plasma membrane on the surface of many epithelial cells (reviewed by Alberts et al., 2002). The core of a microvillus is formed by a bundle of parallel actin filaments cross-linked by actin-bundling proteins such as villin and fimbrin (Fig. 1A). The plus ends of the actin filaments are at the tip of the microvillus, and the actin bundles in the microvillus core extend down into the cell and are rooted in the terminal web, where they are linked together by a complex set of proteins. Actin filaments in the microvillus core are constantly renewed through treadmilling (Rzadzinska et al., 2004). This basic microvillus structure is used by different cell types to carry out different functions. One prominent example is the brush border that lines the apical surface of intestinal epithelial cells in humans (reviewed by Louvard et al., 1992). These tightly packed microvilli maximize the surface area of the epithelial cells for absorption. Another example are the stereocilia found in the mammalian inner ear (reviewed by Brown et al., 2008). Stereocilia are specialized microvilli located on the apical surface of inner ear hair cells that participate in the mechanotransduction process which enables hearing.

As shown above, the basic microvillus structure is used by different cell types for different functions, hence it is important to study its morphogenesis and regulation. For my research, I use the Drosophila ovary as a model system to study microvillus morphogenesis and regulation. During stage 10 of Drosophila oogenesis, epithelial follicle cells surrounding the developing oocyte display on their apical surface a prominent microvillus brush border (Fig. 1C-D; Mahowald and Kambysellis, 1980). Our lab has identified Drosophila Cadherin 99C (Cad99C) to be a critical regulator of microvillus morphogenesis in follicle cells (D’Alterio et al., 2005; Schlichting et al., 2006). Loss of Cad99C results in short and disorganized microvilli, whereas overexpression of Cad99C increases microvillus length.

The mammalian orthologue of Cad99C is protocadherin 15 (Pcdh15). Like Cad99C, Pcdh15 is involved in organizing microvillus-based structures – the stereocilia. Studies of stereocilia development and regulation have led to the identification of a group of genes that, when mutated, cause Usher syndrome type I, the most frequent cause of combined deafness and blindness in humans (reviewed by Reiners et al., 2006). In addition to Pcdh15, the other Usher type I genes
Figure 1 Microvillus based structures.

(A) Intestinal microvillus structure. The core of an individual microvillus consists of parallel bundles of actin filaments, cross-linked by proteins such as villin and fimbrin. The F-actin core is tethered to the plasma membrane core by proteins such as myosin-I. The fast growing end (+ end) of F-actin is inserted at the tip of a microvillus. In intestinal microvilli, the actin filaments extending down from the tips are inserted in the apical cytocortex of the cell, called the terminal web. (B) Stereocilium structure. The core of each stereocilium is similarly composed of parallel F-actin, bundled by cross-linking proteins. However, stereocilia show differential elongation of the F-actin core. The + ends of F-actin are at the tips of the stereocilia, the – end is inserted into the cuticular plate. Adjacent stereocilia are connected to each other by fibrous links. (C) A stage 10A Drosophila egg chamber. The microvillus brush border lines the apical side of epithelial follicle cells surrounding the oocyte. (D) A magnification of the boxed area of C showing microvilli of three follicle cells. The microvillus brush border, which is formed by the apical plasma membrane, faces the oocyte.
A

protein linking F-actin to plasma membrane

terminal web proteins cross-linking F-actin in the apical cytocortex

B

+ end of F-actin cross-linking proteins plasma membrane cuticular plate

C

oocyte

nurse cells

microvillus brush border

D

oocyte

follicle cells

microvilli

apical membrane

basolateral membrane
are cadherin 23, myosin VIIA, Harmonin and SANS. The Usher proteins are thought to participate in an interactome, in which harmonin and SANS act as scaffold proteins integrating all Usher molecules into one protein network. All Usher proteins except SANS have been shown to interact with Pcdh15.

Fly homologues of the two Usher proteins Myosin VIIA and Cadherin 23 (Cdh23), Drosophila Myosin VIIA (MyoVIIA) and Cadherins 74A and 87A (Cad74A and Cad87A), respectively, are also expressed in the Drosophila ovary. Like Cad99C, they are associated with the apical microvillus brush border of follicle cells. Since Cad99C and Pcdh15 seem to have evolutionarily conserved functions in microvillus morphogenesis, and Pcdh 15 has been shown to interact with myosin VIIA and Cdh23, Cad99C may potentially interact with MyoVIIA and Cad74A and Cad87A.

The goal of my research is to further understand the function of Cad99C as a microvillus regulator by identifying its interacting partners, potentially Myosin VIIA, Cad74A and Cad87A, and to further characterize their interactions. The interactions between Cad99C and its potential interactors will be studied both at the molecular and functional levels.

1 Microvillus-based structures

1.1 Intestinal microvilli

The microvillus brush border that lines the apical surface of the intestinal epithelial cells maximizes the surface area of these absorptive cells for nutrient uptake (reviewed by Bement and Mooseker, 1996; Louvard et al., 1992). The intestinal brush border has two distinct regions: thousands of densely packed microvilli that are perpendicular to the apical plasma membrane and the terminal web, which is beneath the microvilli in the apical cytoplasm of the epithelial cells (reviewed by Bement and Mooseker, 1996; Louvard et al., 1992). The core of a microvillus is composed of ~20 actin filaments that are parallel to each other and are of uniform length and width. These actin filaments have the same polarity, with their barbed or plus ends at the tip of the microvilli. From the tip, the actin filaments extend down to the cell, and their rootlets are embedded in the terminal web, which is a complex meshwork of actin filaments cross-linked by proteins such as spectrin and myosin II (reviewed by Alberts et al., 2002; Chhabra and Higgs 2007; Louvard et al., 1992). The actin filaments in the core of the microvillus are linked together
by actin-bundling proteins villin, fimbrin and the smallest isoform of espin. The core actin filaments are tethered to the plasma membrane by a protein complex composed of myosin I and several Ca\textsuperscript{2+} binding calmodulin molecules (reviewed by Alberts et al., 2002; Chhabra and Higgs 2007; Louvard et al., 1992). Another example of microvillus-based structure is the stereocilium, located within the inner ear.

1.2 Inner ear hair cell stereocilia

In the vertebrate inner ear, stereocilia are responsible for converting mechanical stimuli such as sound waves into nerve signals, which are processed by the brain, in a process called mechanotransduction (reviewed by Brown et al., 2008). Sound waves are collected by the outer ear, and as they enter the middle ear, they vibrate the chain of ossicles. The vibration is then transmitted to the inner ear, where mechanotransduction occurs. There are two compartments within the inner ear: 1) the vestibular labyrinth that senses gravity and movement and 2) the cochlea which transduces sound. The cochlea houses a neuroepithelium called the organ of Corti, where hair cells are located (reviewed by Brown et al., 2008; Petit and Richardson, 2009). The hair cells are given the name because of the finger-like extensions on their apical surface. These finger-like extensions are stereocilia, which are specialized derivatives of microvilli (Fig. 1 B).

Similar to intestinal microvilli, stereocilia consist of parallel bundles of actin filaments with barbed ends at the tip of the stereocilia (Tilney et al., 1983). On the surface each hair cell, the stereocilia are arranged into 3 to 4 rows increasing in height. This polarity is important for mechanotransduction – deflections of stereocilia towards the tallest row increases the mechanotransduction current, whereas deflections in the other direction decreases the current (Shotwell et al., 1981). Stereocilia of the three rows of outer hair cells adopt a V or W configuration, whereas the stereocilia of the sole row of inner hair cells are flatter, more curved, and are U shaped. The stereocilia of one hair cell form a hair bundle (reviewed by Petit and Richardson, 2009).

1.3 Brush border microvilli and stereocilia compared

Stereocilia are different from intestinal microvilli in several ways (reviewed by El-Amraoui and Petit, 2005; Frolenkov et al., 2004). First, stereocilia are wider and taller (Tilney et al., 1992). Each hair bundle contains 50-450 stereocilia and each stereocilium in turn contains from 20 to 300 actin filaments, 1 to 120 µm in length (Tilney et al., 1992).
Secondly, not all actin filaments of stereocilia are inserted into the apical cytocortex. Only the centrally located actin filaments are inserted into the cuticular plate which consists of a meshwork of actin filaments that are cross-linked by α-actinin, fodrin and fimbrin (DeRosier and Tilney, 1989; Slepecky and Ulfendahl, 1992; Zine et al., 1995). Most actin filaments of the stereocilia end at the junction between the apical protrusions and the cell body, where stereocilia taper. The tapering provides flexibility to the protrusion, so that it can deflect in response to mechanical stimuli (reviewed by Vollrath et al., 2007).

Third, the actin filaments in stereocilia are cross-linked by espin and fimbrin, not villin (Daudet and Lebart, 2002; Tilney et al., 1989; Zheng et al., 2000). However, the actin-bundling protein espin plays similar roles in both structures. Functional studies of espin showed that in addition to its actin-bundling properties, it regulates the length of both stereocilia and microvilli (Loomis et al. 2003; Rzadzinska et al., 2005). Rzadzinski et al. (2005) analyzed the hair bundles of the jerker mouse, in which a frameshift mutation disrupts the C-terminal actin-binding domain of the espin protein (Zheng et al., 2000), and found that mutant stereocilia were thinner than wild-type stereocilia and progressively shortened. To see whether increasing espin levels can lengthen stereocilia, Rzadzinska et al. (2005) overexpressed espin-GFP in rat inner ear hair cells and found that mutant stereocilia were ~1.5 times longer compared to controls. This is consistent with the effect of gain-of-function of espin on epithelial cell microvilli, where overexpression of espin resulted in an overgrowth of microvilli (Loomis et al., 2003). However, the mechanism of espin-mediated elongation of stereocilia remains unknown.

Lastly, in contrast to microvilli, stereocilia are connected to each other by fibrous extracellular links (reviewed by Brown et al. 2008; Petit and Richardson, 2009). These extracellular links play important roles in the development and function of the hair bundle. The types of links and the position of the different types of links change during the course of development. Some hair bundle links are only transient during development, they include transient lateral links, ankle links, shaft connectors (in the outer hair cells, shaft connectors are maintained throughout adulthood) and kinociliary links. The ones that persist into adulthood include the horizontal top connectors and tip links (reviewed by Brown et al., 2008; Petit and Richardson, 2009). All links connect stereocilia horizontally and there are numerous of each type when they appear, except for the tip link, which connects each stereocilium at its tip to the next taller stereocilium at an angle. Thus there is only one tip link per pair of stereocilia. Except for the tip link, the exact
function of each hair bundle link is still unclear, although it is generally believed that they play essential roles in hair bundle cohesion. The tip link, specifically, is thought to be a gating spring that controls the opening and closing of mechanically gated ion channels (Pickles et al., 1984; Furness and Hackney, 1985). The tip link will be discussed in greater detail in Section 2.

1.4 Microvillus brush border in the *Drosophila* ovary

During *Drosophila* oogenesis, a microvillus brush border is found on the apical surface of epithelial cells (called follicle cells) surrounding the oocyte (Fig. 1C-D; D’Alterio et al., 2005). The microvillus brush border is most prominent at stage 10A, and reaches a maximum length of 2-3 µm. During this stage, follicle cell microvilli are in contact with microvilli from the oocyte (Mahowald and Kambysellis, 1980). Research in our lab has shown that proper microvillus development is needed for proper eggshell formation. One regulator of follicle cell microvilli is Cad99C (discussed later).

The mammalian orthologue of Cad99C is Pcdh15 (D’Alterio et al., 2005), which is involved in organizing stereocilia (Lefevre et al., 2008). Because stereocilia are the mechanosensitive organelles of the inner ear, it is not surprising that a disruption of the structural integrity of stereocilia in the inner ear hair bundle as a result of *Pcdh15* mutations leads to deafness (reviewed by Reiners et al., 2006).

2 Usher proteins

2.1 Protocadherin 15

Mutations in the *Pcdh15* gene cause Usher Syndrome Type I (Ahmed et al., 2001). *Pcdh15* encodes a protocadherin with 11 cadherin domains, one transmembrane domain, and a short cytoplasmic tail with two proline rich regions and a class I PDZ-binding motif (PBM) at its C-terminus (Fig. 2A; Alagramam et al., 2001). There are three alternatively spliced isoforms, CD1, CD2 and CD3. Each isoform has a unique sequence for the cytoplasmic tail and the C-terminus, however, they all include a class I PBM (Ahmed et al., 2006). A fourth isoform is found in the mouse inner ear that lacks the transmembrane domain and the cytoplasmic tail. It is likely a secreted isoform of Pcdh15. As discussed in more detail in the next chapter, Pcdh15 interacts with Cadherin 23 (Cdh23) to form the tip link in mature hair cells. Similar to Cdh23, Pcdh15 localizes to kinociliary links and transient lateral links in developing hair cells of rats, mice and
Figure 2 Protein structures of three Usher proteins, protocadherin 15 (Pcdh15), cadherin 23 (Cdh23), and myosin VIIA, and their respective *Drosophila* homologues.

(A) Pcdh15 and Cad99C have a similar protein structure. They both contain 11 extracellular cadherin domains (CD, shown in blue), one transmembrane domain and a cytoplasmic domain. Cad99C and Pcdh15 show 30% identity across the extracellular cadherin domains. The cytoplasmic region is not conserved, except for the PDZ domain binding site (PBS) at their C-terminus. (B) Cdh23, Cad74A, and Cad87A are characterized by many repeats of cadherin domains in the extracellular region. Cdh23 has a PBS at its C-terminus, while Cad74A and Cad87A are also predicted to have a PBS at their C-terminus, though not confirmed. (C) Both human and fly Myosin VIIA have a unique FERM domain. The third subdomain (F3) of the FERM domain of both proteins has diverged from F3 of other FERM proteins. It is termed the MyTH7 domain.
A

Cad99C (1706 aa)  
Pcdh15 (1943 aa)  

30% similarity across the 11 CDs

PDZ domain binding site

potential Ca\(^{2+}\) binding site

B

Cad74A (1820 aa)  
Cad87A (1975 aa)  

putative PDZ domain binding site

C

Drosophila myosin VIIA (2167 aa)  
Homo myosin VIIA (2216 aa)
guinea pigs (Kazmierczak et al., 2007). Because both Cdh23 and Pcdh15 localize to kinociliary and transient lateral links during hair cell development, it is possible that the two molecules interact in a heterophilic manner, similar to their interaction at the tip link (Kazmierczak et al., 2007).

The C-terminal PBM of Pcdh15-CD1 can interact with two other USHI proteins: myosin VIIA and harmonin (Adato et al., 2005; Senften et al., 2006). To look for an interaction between myosin VIIA and Pcdh15, Senften et al. (2006) performed co-immunoprecipitation (co-IP) experiments using two truncated isoforms of myosin VIIA fused to GFP: a fragment containing the myosin VIIA tail and another containing only the SH3 domain and MyTH4 domain. GFP-tagged myosin VIIA isoforms and full length Pcdh15 were expressed in HEK293 cells. IP was performed with an anti-GFP antibody. Subsequent SDS-PAGE and western blot analysis using an anti-Pcdh15 antibody demonstrated that Pcdh15 co-IPed with both myosin VIIA peptides. Next, Senften et al. (2006) showed that the myosin VIIA-Pcdh15 interaction was direct by performing GST pull downs. Furthermore, the SH3 domain of myosin VIIA alone was sufficient to pull down in vitro translated Pcdh15 cytoplasmic tail. Together, these results suggested that myosin VIIA interacts with Pcdh15 directly and that the interaction was mediated by the SH3 domain of myosin VIIA. The authors extended the in vitro interaction studies further by co-expressing in cell lines either myosin VIIA and a recombinant protein with EC and TM domains of E-cadherin fused to the Pcdh15 cytoplasmic tail (Ecad-Pcdh15cyto) or myosin VIIA and a fusion protein between EC and TM domains of E-cadherin and a HA tag (Ecad-HA). Immunofluorescence revealed that Ecad-Pcdh15cyto and Ecad-HA localized to cell-cell contacts and myosin VIIA was only recruited to cells expressing Ecad-Pcdh15cyto. Lastly, in mouse cochlear hair cells, distribution patterns of myosin VIIA and Pcdh15 partially overlap, and the localization of myosin VIIA and Pcdh15 in stereocilia was disrupted in Pcdh15-deficient Ames Waltzer and myosin VIIA-deficient Shaker-1 mice, respectively (Senften et al., 2006).

### 2.2 Cadherin 23

Mutations in the Cad23 gene are responsible for USHID (Bolz et al., 2001). Cdh23 is a non-classical cadherin with 27 extracellular cadherin domains, one transmembrane domain and a short cytoplasmic tail (Fig. 2B; Bolz et al., 2001). There are three Cdh23 isoforms, A, B and C. Isoform A (Cdh23 +68) has an insert encoded by exon 68 in the cytoplasmic tail, whereas
isoform B (Cdh23-68) does not (Siemens et al., 2002). Isoform C consists only of the cytoplasmic tail, lacking cadherin domains and the transmembrane domain (Lagziel et al., 2005). Isoform C may compete with A and B isoforms for cytoplasmic binding partners, thereby regulating signalling pathways in the cytoplasm (Lagziel et al., 2005; reviewed by Reiners et al., 2006). Cdh23-A is exclusively expressed in inner ear hair cells, while Cdh23-B is broadly expressed in other tissues such as heart, kidney, spleen, brain, and retina (Siemens et al., 2002).

The cytoplasmic tail of Cdh23 contains two putative PDZ domain binding motifs (PBM). A class I PBM (X8S/T8X8V) is found at the C-terminus. Isoform B contains an additional internal PBM, which is disrupted in isoform A by the 35 amino acid insert encoded by exon 68. Cdh23 binds to the PDZ1 and PDZ2 domains of harmonin via its internal and C-terminal PBM, respectively (Boeda et al., 2002; Siemens et al., 2002).

Cdh23 is a component of several linkages during hair cell development in mice (reviewed by Muller, 2008). Localization studies at both light and electron microscopic resolutions revealed that Cdh23 localizes to kinociliary links and transient lateral links (Lagziel et al., 2005; Kazmierczak et al., 2007; Michel et al., 2005; Siemens et al., 2004). However, the molecular composition of these linkages, as well as the spatial arrangement of Cdh23 in these linkages still need to be deciphered (Muller, 2008).

In the mature cochlear hair cell, Cdh23, together with Pcdh15 make up the tip link (Ahmed et al., 2006; Kazmierczak et al., 2007; Siemens et al., 2004). Previously, conflicting results were reported by several labs on the localization of Cdh23 and Pcdh15. To verify the localization of the two proteins at the tip link, Kazmierczak et al. (2007) generated three different antibodies specific to different regions of Cdh23 and Pcdh15: cadherin domains 1/2 and 15/16 of Cdh23, and cadherin domain 1 of Pcdh15. Immunostaining experiments showed that Cdh23 and Pcdh15 localized to tip links in mouse, rat and guinea pig hair cells. Subsequently, the three antibodies were used in immunogold labelling and electron microscopy analyses to determine the distribution of various cadherin domains of Cdh23 and Pcdh15 along the tip link filament. The results obtained placed Cdh23 and Pcdh15 at the upper and lower parts of the tip link, respectively. Furthermore, the immunolocalization studies showed that the N-termini of Cdh23 and Pcdh15 colocalized at the same position along the tip link, suggesting that Cdh23 and Pcdh15 interact at their N-termini via the first cadherin domain, similar to how classical cadherins interact (Halbleib and Nelson, 2006). The authors further tested the mode of
interaction of Cdh23 and Pcdh15 by conducting biochemical experiments and TEM analyses. Biochemical experiments using purified recombinant cadherin domains of Cdh23 and Pcdh15 confirmed that the two molecules can interact via their N-termini. TEM images of the purified recombinant proteins revealed that the EC domains of Cdh23 and Pcdh15 intertwine to form homodimers. In addition, Cdh23 homodimers interact in trans with Pcdh15 homodimers at their N-termini. The resulting filaments formed by the two molecules were ~180 nm in length, which agreed with reported tip link dimensions (Furness et al., 2008).

Recently, two Cdh23 interaction partners were isolated from two independent yeast two hybrid screens: MAGI-1, a membrane-associated guanylate kinase protein and EHD4, a member of the C-terminal EH (eps15 homology) domain containing protein family involved in endocytosis (Senpgupta et al., 2009; Xu et al., 2008). The Cdh23-MAGI-1 and Cdh23-EHD4 interactions were further confirmed by immunocolocalization studies as well as in vitro binding experiments. Xu et al., (2008) proposed MAGI-1 to be another scaffolding protein in the stereocilia because of the multi-protein interaction domains that exist in the protein as well as its ability to bind the C-terminal PBM of Cdh23. Although harmonin can also bind to the C-terminal PBM of Cdh23, it binds with a much lower affinity (Boeda et al., 2002; Siemens et al., 2002). More data are needed to test whether MAGI-1 could act as scaffolding protein in stereocilia. The functional significance of the Cdh23-EHD4 interaction is yet to be determined. Perhaps it is involved in the trafficking or localization of Cdh23 in cochlear hair cells (Senpguta et al., 2009).

### 2.3 Myosin VIIA

Myosin VIIA is encoded by USHIB (Weil et al., 1995). Myosin VIIA contains a motor head domain at its N-terminus, followed by a neck region containing 5 isoleucine glutamine (IQ) repeats, and lastly a tail region that serves to anchor the molecule at a specific cellular location (Fig. 2C). The motor head domain contains both actin- and ATP-binding sites, which allows MyoVIIA to walk along actin filaments toward the barbed end (Inoue and Ikebe, 2003). The 5 IQ motifs at the neck region interact with myosin light chains - calmodulin (Bahler and Rhoads, 2002; Udovichenko et al., 2002). The tail begins with a short coiled-coil domain that is thought to mediate homodimerization. It is then followed by 2 MyTH4 (myosin tail homology 4) domains, a SH3 (src homology 3) domain and 2 FERM (band 4.1, ezrin, radixin, moesin) domains (reviewed by Sellers, 2000). The function of the MyTH4 domain is unclear. The FERM
domain is thought to mediate the interaction between the cytoskeleton to the plasma membrane (reviewed by Tepass, 2009). In the cochlea, myosin VIIA is predominantly located in the stereocilia, although it is also found along the lateral membrane, in the cuticular plate and in the synaptic region (reviewed by Reiners et al., 2006).

Studies in myosin VIIA-deficient mice strongly suggest that myosin VIIA is necessary for normal stereocilia bundle organization. In shaker-1 mice that lack MyoVIIA, disorganized and fragmented hair bundles for both the inner and outer hair cells were observed as early as E17.5 and E18.5, respectively (Lefevre et al., 2008). Instead of forming a V-shaped hair bundle, stereocilia were clumped together, and 2-3 clusters were often observed on the apical surface of a hair cell. In addition, the shaker-1 mutant hair bundles displayed misoriented kinocilia and elongation defects (Lefevre et al., 2008; Self et al., 1998).

Myosin VIIA is important for the localization of harmonin and Pcdh15 to the stereocilia. Harmonin and Pcdh15 are mislocalized in hair bundles of the shaker-1 mice, and myosin VIIA had been shown to interact with these two proteins in vitro (Boeda et al., 2002; Senften et al., 2006). Recently, there is evidence that myosin VIIA may act as a more general transporter of stereociliary proteins. Immunofluorescence analysis conducted on hair bundles of shaker-1 mice, that lack myosin VIIa because of a nonsense mutation in the motor head domain, revealed that components of the ankle link – usherin, vezatin, and Vlgr1 were absent in the basal region of stereocilia where ankle links are normally located. Instead they formed clumps at the apical surface of hair cells (Michalski et al., 2007). In the same study, the authors showed that the C-terminal MyTH4-FERM fragment of myosin VIIA can directly bind to GST-fusion proteins containing the cytoplasmic domain of either Vlgr1 or usherin. Direct interactions between myosin VIIA tail and vezatin had been shown previously (Kussel-Andermann et al., 2000). Taken together, these data suggest that myosin VIIA, acting as a molecular motor, transports USH proteins to their proper locations in the stereocilia (Michalski et al., 2007; Saihan et al., 2009).

Recently, a novel role of myosin VIIA in regulating stereocilia lengths was proposed. Prosser et al. (2008) generated mosaics of wild-type and myosin VIIA-deficient hair cells in the inner ear epithelium of mice. When compared side by side, the tallest stereocilia lacking myosin VIIa are significantly longer than their wild-type counterparts but fewer in number. The elongation of
myosin VIIA-deficient stereocilia was not due to a decrease in the depolymerization rate at the pointed ends (Prosser et al., 2008). When cytochalasin D, an actin polymerization inhibitor, was added to the sensory epithelia mosaic for myosin VIIA expression, myosin VIIA-deficient stereocilia shortened faster, indicating faster actin depolymerization. While myosin VIIA may be involved in increasing actin polymerization at the barbed ends, more experiments are needed to test this hypothesis.

Other roles have been proposed for myosin VIIA such as organizing the spatial distribution of other interstereociliary links as it can interact with the scaffold protein harmonin and transport harmonin-protein complexes to their appropriate positions within the stereocilium. Furthermore, by linking the actin cytoskeleton to the plasma membrane, myosin VIIA could influence the development and maturation of stereocilia (reviewed by El-Amraoui and Petit, 2005; Petit and Richardson, 2009). However, direct evidence supporting these models are lacking. More data are awaiting to elucidate the function(s) of myosin VIIA in inner ear hair cells.

3 *Drosophila* homologues of Usher proteins: Cad99C, Cad74A, Cad87A and MyoVIIA

As mentioned above, Cad99C, Cad74A and Cad87A, and MyoVIIA are the homologues of Pcdh15, Cdh23, and myosin VIIA, respectively. That the Usher proteins functionally interact to regulate stereocilium morphogenesis raises the possibility that the *Drosophila* homologues may participate in a common process in controlling microvillus morphogenesis. This section is dedicated to discussing these *Drosophila* proteins. Because they will be mostly discussed in terms of their functions during *Drosophila* oogenesis, and the *Drosophila* ovary was used as a model system in my research, below an overview of *Drosophila* oogenesis will be given preceding the discussion of these proteins.

3.1 Overview of *Drosophila* oogenesis

The *Drosophila* ovary is composed of ~16 parallel ovarioles (King, 1970; Spradling, 1993; Wu et al., 2008). An ovariole is the functional unit of the ovary and consists of a string of egg chambers of different developmental stages. An egg chamber or follicle consists of germ cells (nurse cells and the oocyte) surrounded by a monolayer of somatic epithelial cells (or follicle cells). Egg chambers are made at the anterior tip of the ovariole, and move toward the posterior
end as they mature. The ovariole is composed of two regions: the germarium at the anterior end, characterized by the presence of stem cells and where the basic structure of an egg chamber is being formed; and the larger, more posterior vitellarium, where the egg chambers grow and mature. There are 14 stages of oogenesis, subdivided by King (1970) based on morphological criteria (see Fig. 3 for stages 10-13). A stage 1 egg chamber is formed in the germarium with germ cells surrounded by ~80 follicle cells (King, 1970; Mahowald and Kambysellis, 1980; Spradling, 1993). Stage 2 egg chambers bud off from the germarium and enter the vitellarium. During stages 2-7, the oocyte grows at the same rate as the nurse cells, thus they are of the same size (King, 1970). During stages 8 to 10, the oocyte grows at a greater rate than nurse cells, due to uptake of yolk proteins synthesized by follicle cells and fat bodies (King, 1970; Mahowald and Kambysellis, 1980). By stage 10A, the oocyte occupies half of the egg chamber (Fig. 3A). Throughout oogenesis, nurse cells transport their mRNAs and proteins to the developing oocyte (King, 1970). The transport is slow initially, but increases dramatically from stages 10B to 12 (Fig. 3C-E). Most of the nurse cell cytoplasm is transferred in 30 minutes at stage 11, and the transfer is complete by stage 12 (Cooley et al., 1992; Spradling, 1993). Subsequently, during stages 13-14, the nurse cells undergo apoptosis while the oocyte matures for the remainder of oogenesis (Cavalier et al., 1998).

The follicle cells that overlie the developing oocyte and nurse cells form a cuboidal epithelium until stage 8 of oogenesis (reviewed by Horn-Badovinac and Bilder, 2005). Starting from stage 9, follicle cells undergo a series of morphological changes and migrations, and secrete eggshell proteins, all of which are important for the formation of a proper eggshell. At stage 9, approximately 600 follicle cells migrate posteriorly to cover the oocyte and they become columnar in shape (reviewed by Horn-Badovinac and Bilder, 2005). The remaining ~50 follicle cells stretch to cover the 15 nurse cells at the anterior. They have a squamous morphology. By stage 10A, a sharp boundary between the columnar and squamous follicle cells can be seen at the nurse cell-oocyte border (Fig. 3A; reviewed by Horn-Badovinac and Bilder, 2005). Stage 10B egg chambers are characterized by the inward migration of the most anterior columnar follicle cells (also called centripetal follicle cells) at the nurse cell-oocyte border to close the anterior end of the oocyte (Fig. 3B). From stages 10B to 14, follicle cells become thinner as they secrete the eggshell proteins (Fig. 3C-E). The follicle cells undergo apoptosis when the eggshell formation is completed (Nezis et al., 2002).
Figure 3 Stage 10 to stage 13 of Drosophila oogenesis.

(A) At stage 10A, the oocyte occupies half of the egg chamber. The follicle cells overlying the oocyte are columnar in shape, those surrounding the nurse cells are squamous. At this stage, a prominent microvillus brush border lines the apical surface of follicular epithelial cells that cover the oocyte (red outline). (B) At stage 10B, the follicle cells surrounding the oocyte have a cuboidal morphology. The most anterior follicle cells migrate inwards at the nurse cell-oocyte border to close the anterior end of the oocyte. The vitelline bodies situated in between microvilli start to fuse (dotted blue line around the oocyte). (C) At stage 11, the oocyte occupies ~3 quarters of the egg chamber. By now, vitelline bodies have fused into a continuous layer. Follicle cells flatten over the oocyte. They start to secrete chorion proteins. (D) At stage 12, the oocyte is fully grown as all nurse cell cytoplasm has been transferred to the oocyte. The dorsal appendages extend from either side of the oocyte. (E) At stage 13, the oocyte has reached its maximum volume. The endochorion is formed (solid blue line).
3.2 Cadherin 99C

Cad99C is the orthologue of the mammalian Pcdh15 (D’Alterio et al., 2005; Schlichting et al., 2006). Cad99C encodes a non-classical cadherin with 11 extracellular cadherin (EC) domains, one transmembrane domain, and a cytoplasmic tail containing a class I PBM at its C-terminus (Fig. 2A). The 11 EC domains show 30% identity to the EC domains of Pcdh15. The cytoplasmatic tail is not conserved between the two molecules except for the PBM. Cad99C seems to play an evolutionarily conserved role in microvillus morphogenesis (D’Alterio et al., 2005; Schlichting et al., 2006). As discussed above, Pcdh15 is localized to stereocilia, which are actin-based protrusions in the inner ear. It is required for stereocilia cohesion and growth. In the Drosophila ovary, Cad99C is expressed by the follicle cells overlying the oocyte (D’Alterio et al., 2005; Schlichting et al., 2006). It is localized to the apical microvillus brush border of follicle cells and is distributed throughout the plasma membrane of each microvillus. Cad99C is involved in microvillus biogenesis and regulation. Mutant alleles of Cad99C caused severe morphological defects in microvilli (D’Alterio et al., 2005; Schlichting et al., 2006). In wild-type follicles, microvilli form a regular pattern on the apical side of follicle cells. The microvilli of one follicle cell are longest in the centre, shorter in the cell periphery. Looking down on the apical surface of the follicle cells, microvilli can be seen forming regular tufts. The pattern reflects the honeycomb-like pattern of follicle cells (D’Alterio et al., 2005; Schlichting et al., 2006). In contrast, the regular microvillus pattern is lost in Cad99C mutant follicles. The apical protrusions appear shorter and more sparse. In some cases, no apical protrusions were produced. In support of this, F-actin was largely missing in the space between the follicle cells and the oocyte. Top view of the apical surface of mutant follicle cells also showed an irregular distribution of microvilli (D’Alterio et al., 2005; Schlichting et al., 2006). The microvilli formed in Cad99C mutants showed similar morphological defects to the splayed stereocilia found in the Pcdh15 mutants.

Using Cad99C as a marker for microvilli, D’Alterio et al. (2005) found that microvilli undergo dynamic changes in morphology. A microvillus brush border was first detected at stage 7 of Drosophila oogenesis. The height of the brush border increases in stages 7-10, reaching its maximum height at stage 10A (Fig. 4A-A”). After stage 10B, microvilli seem to regress, and remain very short until the end of oogenesis (Fig. 4B-B”). The regression of microvilli coincides with the formation of the vitelline membrane. During stages 8-10, follicle cells secrete
components of the vitelline membrane in vesicles into the space between follicle cells and the oocyte (reviewed by Waring, 2000). These vesicles are called vitelline bodies. At late stage 10B, the vitelline bodies fuse to form a continuous layer called the vitelline membrane, the first eggshell layer to be deposited. Vitelline bodies in Cad99C mutants are irregular in size, shape and distribution (D’Alterio et al., 2005). Consequently, the vitelline membrane was unevenly deposited and varied in thickness in these mutants. Numerous holes were observed, resulting in dessication of eggs laid by Cad99C mutant females. These observations explained why Cad99C mutant females were sterile (D’Alterio et al., 2005; Schlichting et al., 2006).

Gain-of-function analysis showed that Cad99C is a positive regulator of microvillus length. When full-length Cad99C was overexpressed in follicle cells, an overgrowth of follicle cell microvilli resulted (D’Alterio et al., 2005; Schlichting et al., 2006). Moreover, the cytoplasmic tail of Cad99C is dispensable for promoting microvilli overgrowth as overexpressing just the extracellular cadherin domains of Cad99C also resulted in long microvilli, similar as expressing Cad99C full length (D’Alterio et al., 2005).

3.3 Cadherin 74A and Cadherin 87A

Cad74A encodes a non-classical cadherin containing 14 cadherin domains, one transmembrane domain, and a cytoplasmic domain (Fig. 2B; Hill et al., 2001; Zartman et al., 2008). Sequence analysis revealed that Cad74A is a homologue of vertebrate Cdh23 (Hwang and Godt, unpublished). Unlike Cad99C, Cad74A was found to mediate Ca\(^{2+}\)-dependent adhesion in cell cultures (Lovegrove et al., 2006). Cad74A is also expressed in the Drosophila ovary. However, its expression pattern is more complex compared to that of Cad99C. Cad74A is expressed in the follicular epithelial cells around the oocyte from stage 10B and onwards, and at stage 14, the protein can still be detected. However, Cad74A is not expressed uniformly throughout the follicular epithelium. At stage 11, its expression is downregulated in two dorsal anterior patches of cells that are the primordia of the dorsal appendages (DA) (Hwang and Godt, unpublished; Zartman et al., 2008), suggesting that Cad74A might play a role in dorsal appendage formation. DAs are tube-like structures at the anterior of an embryo. They facilitate gas exchange during embryonic development (reviewed by Waring, 2000). In follicle cells that express this protein, anti-Cad74A antibodies, raised in our lab as well as in another, revealed that at stage 10B when Cad74A is first expressed, it is localized to the apical membrane and microvilli (Hwang and
Figure 4 Microvillus and vitelline membrane formation during late stages of *Drosophila* oogenesis.

(A and B) are schematic drawings of stage 10B and stage 12 egg chambers, respectively. (A’, B’) Anti-Cad99C antibody reveals the microvillus border on the apical surface of follicle cells at stage 10B. The brush border begins to regress in late stage 10B and onwards. Only short microvilli can be seen at stage 12 (B’). (A” and B”’) are Nomarski images of (A’ and B’), respectively. (A”) At stage 10B, the microvillus brush border can be seen as a stripe pattern band in the Nomarski image. It reflects the positions of the vitelline bodies, separated by microvilli. (B”) By stage 12, vitelline bodies have fused to form a continuous layer – the vitelline membrane. (C) Schematic drawing of a mature egg chamber. The vitelline membrane is the first layer of the eggshell to be synthesized. It is the most proximal layer to the oocyte. (D-D”) are schematic drawings of the formation of the vitelline membrane. (D) At stage 10B, the follicle cells around the oocyte secrete vitelline bodies, which are deposited between the follicle cells and the oocyte, and are located in between microvilli. (D’) The vitelline bodies fuse. (D”) At stage 12, a continuous layer of vitelline membrane is formed. Bars, 10µm. Schematic drawings are drawn after pictures provided by D. Godt.
A. Stage 10B
- Follicular epithelium
- Oocyte
- Nurse cells

B. Stage 12
- Oocyte
- Follicle cells

C. Mature egg
- Dorsal appendage
- Operculum
- Micropyle

Annotations:
- Cad99C
- Vitelline membrane
- Inner chorion layer
- Endochorion
- Exochorion
- Chorion
Godt, unpublished; Zartman et al., 2008), suggesting a possible function of Cad74A in microvillus morphogenesis. From stage 11 and onwards, Cad99C and Cad74A continue to be present in apical microvilli. However, the expression of Cad99C decreases progressively as oogenesis proceeds while Cad74A remains highly expressed during these late stages (D’Alterio et al., 2005; Hwang and Godt, unpublished; Zartman et al., 2008).

Cad74A is involved in eggshell morphogenesis. A fraction of eggs (17%) laid by Cad74A-null females displayed severe DA defects (Zartman et al., 2008). These eggs either lacked DAs or DAs were deformed. In addition, the DAs varied in length. Experiments conducted by Zartman et al. (2008) suggested that the repression of Cad74A by Broad (BR) in the roof cells (dorsal appendage primordia) during oogenesis supports proper DA formation. The authors studied the expression of Cad74A in roof cells that were homozygous for a null allele of broad and found that Cad74A was ectopically expressed. In addition, overexpression of the transcription factor pointed which repressed BR expression, led to strong expression of Cad74A (Zartman et al., 2008). Furthermore, when Cad74A was ectopically expressed in the roof cells, the following were observed: 1) DAs of Cad74A overexpressing egg chambers are shortened and flattened compared to controls. 2) In Cad74A overexpressing egg chambers, large aggregates of ectopic protein accumulated in the apical half of the roof follicle cells. 3) At stage 13/14, when DAs had formed in control egg chambers, the roof cells of Cad74A overexpressing egg chambers failed to migrate towards the anterior tip of the egg chamber. This is consistent with the flattened morphology observed. All these results strongly suggest that repression of Cad74A by Broad in the roof cells is required for normal DA formation (Zartman et al., 2008).

*Drosophila* Cadherin 87A (Cad87A) is a second homologue of Cdh23 (Fung et al., 2008). It encodes a non-classical cadherin that consists of 15 extracellular cadherin domains, one transmembrane domain and a cytoplasmic domain (Fig. 2B). The expression pattern of Cad87A in the *Drosophila* ovary also raises the possibility that it might function in microvillus regulation, vitelline membrane morphogenesis, as well as dorsal appendage formation during *Drosophila* oogenesis (Chase, M. Sc. Thesis, 2004). Like Cad74A, Cad87A is detected in the follicle cells in large amounts that overlie the oocyte starting from stage 10B of oogenesis. Double immunostaining experiments with anti-Cad99C and anti-Cad87A antibodies suggested that Cad87A is localized to the base of microvilli in these follicle cells (Chase, M.Sc. thesis 2004), suggesting a possible role of Cad87A in microvillus regulation. The apical surface of the
follicular epithelial cells that overly the oocyte continue to display Cad87A during late oogenesis (stages 11-14), a period when the eggshell is formed, suggesting a possible role of Cad87A in eggshell formation. In dorsal appendage primordial cells (two patches follicle cells where Cad74A is downregulated), Cad87A is upregulated (Chase, M.Sc. thesis 2004). Hence, Cad87A seems to be complementary to Cad74A for dorsal appendage formation. Finally, unlike Cad99C and Cad74A, which are only expressed in somatic cells, Cad87A is also detected in the oocyte. However, the function of Cad87A in the oocyte is unclear (Chase, M.Sc. thesis 2004).

3.4 Drosophila Myosin VIIA

*Drosophila* Myosin VIIA (MyoVIIA) is encoded by the *crinkled* locus (*ck*, Kiehart et al., 2004). Its sequence is 61.7% and 58.8% identical to its human and *C. elegans* orthologues, respectively. MyoVIIA encodes a 250 kDa protein. Like its human orthologue, *Drosophila* MyoVIIA is comprised of a N-terminal spectrin-like SH3 domain, a motor head, followed by five isoleucine-glutamine (IQ) motifs in the neck region, and coiled-coil, myosin tail homology 4 (MyTH4), src homology 3 (SH3), and 4.1, ezrin, radixin, moesin (FERM) domains in the tail region (Fig. 2C). Myosin VIIA proteins have a unique FERM domain compared to other proteins that contain FERM domains. In general, FERM domains consist of 3 subdomains – F1, F2, and F3 (reviewed by Tepass, 2009). Together, these 3 subdomains form a cloverleaf-like structure. For myosin VIIA, while F1 and F2 subdomains are conserved with the F1 and F2 subdomains of other FERM proteins, F3 is only conserved among the myosin VII proteins. Kiehart et al. (2004) referred to this last subdomain of the FERM domain of MyoVIIA as the myosin tail homology 7 (MyTH7) domain. In vitro molecular analysis of *Drosophila* MyoVIIA by Yang et al. (2009) and Umeki et al. (2009) showed that the C-terminal MyTH7 domain has autoinhibitory properties. At low actin concentrations, the myoVIIA tail bends back such that MyTH7 binds to the head and the last three IQ motifs in the neck region, thereby inhibiting the Mg\(^{2+}\)-ATPase activity of the motor head domain. In the opposite scenario, at high actin concentrations, the second FERM domain (including the MyTH7 domain) of MyoVIIA binds to actin, thereby unfolding the molecule and alleviating the autoinhibition (Yang et al., 2009).

At the molecular level, it was thought that MyoVIIA acts as a cargo transporter (Watanabe et al., 2006; Yang et al., 2006). Being a cargo transporter requires MyoVIIA to dimerize (i.e. form a two-headed structure) and move processively on actin. Indeed, when MyoVIIA was artificially
induced to dimerize, it showed kinetic properties of a high duty ratio motor (which means it remains bound to actin throughout most of the ATPase cycle), and could take a large number of steps before detaching from F-actin, suggesting that MyoVIIA can function as a cargo transporter (Watanabe et al., 2006; Yang et al., 2006). However, recent electron microscopy analyses conducted by two independent labs showed that MyoVIIA is monomeric (Umeki et al., 2009; Yang et al., 2009). In addition, the coiled-coil domain is too short for it to form a stable dimer. Both groups proposed that perhaps binding to its interacting partner(s) would induce or support dimer formation, and allow MyoVIIA to function as a cargo transporter. Thus it will be important to determine the structure and kinetic properties of MyoVIIA when it is bound to its binding partner(s) (Umeki et al., 2009; Yang et al., 2009).

ck mutants that are homozygous for the null alleles ck^{13} and ck^{7}, die as embryos and larvae, respectively (Kiehart et al., 2004). Adult escapers for these alleles are infertile and very short-lived. In addition, they show a variety of morphological defects in their hairs and bristles of the thorax, head and wing. First, SEM analyses showed that in ck mutants, instead of one hair per cell, numerous hairs were observed for one cell (2-3 for wing hairs and 5-8 for hairs on the rest of the body). Moreover, mutant wing hairs also show branching at their tips. Secondly, bristles on the thorax, head and wing of escapers are short, branched and often twisted. Moreover, abnormally deep and irregular grooves were observed on mutant bristles. Lastly, bristles arising from the antennae, arista, are shorter and more highly branched compared to control aristae. Together, the defects in hairs and bristles seen in ck mutants suggest that MyoVIIA plays important roles in positioning actin bundles that will give rise to hairs and bristles during development (Kiehart et al., 2004; reviewed by DeRosier and Tilney, 2000).

MyoVIIA is needed for hearing in the fly. Studies by Todi et al. (2005; 2008) have demonstrated that ck mutants are deaf because the auditory organ, Johnston’s organ does not develop properly, leading to a disorganized structure in the adult (Todi et al., 2005). Furthermore, MyoVIIA is needed to maintain Johnston’s organ organization in the adult. Removal of MyoVIIA at the adult stage leads to progressive deafness as a result of progressive disorganization of the Johnston’s organ (Todi et al., 2008). As discussed previously, the mammalian myosin VIIA is needed for the structural integrity of hair cell stereocilia, and the Johnston’s organ is functionally related to the inner ear, therefore, MyoVIIA plays a role in auditory organ development and maintenance in both invertebrates and vertebrates (Todi et al., 2005; 2008).
Functional analysis of MyoVIIA in our lab has shown that it is required for microvillus development during *Drosophila* oogenesis (Glowinski, M.Sc. Thesis, 2008). MyoVIIA is detected in follicle cell microvilli as early as stage 9, and remains present in microvilli in the apical surface of follicle cells until late oogenesis (stage 13). At stage 10A, when the microvillus brush border is most prominent, MyoVIIA is enriched in follicle cell microvilli. However, MyoVIIA is only detected in the basal region of microvilli, not throughout the entire length.

In MyoVIIA-deficient egg chambers, microvillus morphology was abnormal (Glowinski, M.Sc. Thesis, 2008). The microvilli of MyoVIIA mutant egg chambers are shorter and thinner compared to wild-type, suggesting a function of MyoVIIA in microvillus length regulation, similar to the function of Cad99C in microvillus morphogenesis. However, MyoVIIA-deficient microvilli differ from Cad99C-deficient microvilli in that those in *Cad99C* mutants were shorter and disorganized.

Because both Cad99C and MyoVIIA are involved in microvillus development during *Drosophila* oogenesis, we wanted to know whether they function together in the same regulatory mechanism that controls microvillus morphogenesis. To look for an interaction between Cad99C and MyoVIIA, immunolocalization studies were conducted to determine the distribution of Cad99C and MyoVIIA in MyoVIIA- and Cad99C-deficient follicle cells, respectively (Glowinski, M.Sc. Thesis, 2008). In *Cad99C* mutant follicle cells, the amount of apical MyoVIIA is reduced. However, it is not certain whether this defect is a result of the loss of Cad99C or of abnormal microvillus morphology. Conversely, in follicle cells that are devoid of MyoVIIA, although the distribution of Cad99C appears to be normal, its amount is significantly reduced in the microvilli. This indicates that MyoVIIA is at least partially responsible for accumulating proper amounts of Cad99C in the microvilli. Together, these results provide the first piece of evidence that Cad99C and MyoVIIA may function in the same regulatory mechanism in controlling microvillus morphogenesis (Glowinski, M. Sc. Thesis 2008).

### 4 Microvilli and vitelline membrane of the *Drosophila* ovary

One of the most important functions of the follicle cells surrounding the oocyte is the secretion of eggshell proteins. As discussed in the previous section, loss of function of Cad99C results in a defective eggshell, and because Cad99C is an essential component of microvilli, this suggests
that microvilli are important for proper eggshell formation. This last section will discuss the interplay between microvilli and the first eggshell layer – the vitelline membrane, the proteins that are involved in their formation, as well as mechanisms of function of these proteins if known.

4.1 The different eggshell layers

The eggshell is formed by components deposited by columnar follicle cells during mid to late oogenesis (reviewed by Waring, 2000). This complex structure has specialized parts that are important for the survival of the mature oocyte and embryo. The specialized parts include the dorsal appendages, micropyle, and operculum at the anterior end of the egg. Dorsal appendages facilitate gas exchange during embryonic development. The micropyle is the structure where sperm enters. Lastly, the operculum is a flat plate where the larva escapes once it hatches (Fig. 4C; reviewed by Waring, 2000).

The eggshell is multilayered (Fig. 4C; reviewed by Waring, 2000). From the oocyte to the outer eggshell surface, five morphologically distinct layers can be identified: the vitelline membrane, a lipid wax layer that is water impermeable and thus prevents dessication of the embryo, an inner chorion layer, an endochorion, and an exochorion. The latter 3 layers are collectively termed the chorion (reviewed by Cavaliere et al., 2008; Waring 2000). The vitelline membrane will be discussed in greater detail in the next section.

4.2 Microvilli and vitelline membrane mophogenesis: players and actions

As discussed in Section 3, microvilli form during stages 7-10 of oogenesis. Concurrent with microvillus development, during stages 9-10, the follicle cells overlying the oocyte are actively engaged in synthesizing and secreting the vitelline membrane – the first eggshell layer to be made (Mahowald and Kambysellis, 1980). During this stage, the vitelline membrane is being deposited as aggregates of vitelline membrane proteins (vitelline bodies) into the extracellular space between the follicle cells and the developing oocyte. Both Nomarski optics and SEM analysis showed that these vitelline bodies, 1 µm in diameter, are situated in between and are separated by microvilli that project from both the oocyte and follicle cells (Fig. 4A-A”; D’Alterio et al., 2005; Mahowald and Kambysellis, 1980). By the end of stage 10, the vitelline
bodies fuse with each other, forming a continuous layer – the vitelline membrane that is 1.7 µm in thickness (Mahowald and Kambysellis, 1980; Margaritis, 1985). However it thins to 0.3 µm by the end of oogenesis (Margaritis, 1985). Meanwhile, the microvillus brush border regresses progressively from stage 10B and onwards. By stage 12, Cad99C staining reveals a very thin band on the apical surface of follicle cells (Fig. 4B; D’Alterio et al., 2005).

Four major structural proteins of the vitelline membrane have been characterized thus far: sV17, sV23, VM32E, and VM34E (reviewed by Waring, 2000). All are small proteins (116-168 amino acids), rich in alanine, proline, serine, and glycine, and contain the VM domain, a highly conserved hydrophobic sequence of 38 amino acids, at their C-termini. Following secretion, the vitelline membrane proteins undergo posttranslational processing and subsequent disulfide cross-linking (Andrenacci et al., 2001; Manogaran and Waring, 2004; Pascucci et al., 1996). For two of these proteins, sV23 and VM32E, the VM domain has been shown to be important for disulfide cross-linking (Andrenacci et al., 2001; Manogaran and Waring, 2004). Recently, a minor constituent of the vitelline membrane, Palisade, has been shown to be essential for vitelline membrane morphogenesis (Elalayli et al., 2008). Palisade differs from the other four vitelline membrane proteins in its protein structure. It is bigger (391 amino acids) and lacks the VM domain. The only similarity is that its central domain is enriched in proline, alanine, and tyrosine, which is also the case for sV23, VM32E and VM34E but not sV17 (Elalayli et al., 2008). To study the role of Palisade in vitelline membrane assembly, Elalayli et al. (2008) generated Palisade-deficient egg chambers and analyzed the vitelline membrane structure using electron microscopy. At stage 10, instead of accumulating regular sized vitelline bodies in an evenly spaced manner, Palisade-deficient egg chambers showed accumulation of vitelline bodies that were irregular in size and shape, and their distribution. Consequently, at stage 11, holes were readily observed in the vitelline membrane during coalescence of the vitelline bodies. Nonetheless, the vitelline membrane recovered when the vitelline bodies had fused completely, giving rise to a continuous structure without gaps, and an intact chorion layer. To study the role of Palisade further, Elalayli et al. (2008) studied the distribution, proteolytic processing and cross-linking of sV17 and sV23 in Palisade-deficient egg chambers. The authors found that in the absence of Palisade, sV17 was abnormally taken up by the oocyte, and that the processing and disulfide cross-linking of sV17 and sV23 were compromised. In conclusion, Palisade is not only needed for the initial assembly of the vitelline membrane, the latter experiments suggest a
second role in coordinating the assembly of other vitelline membrane proteins (Elalayli et al., 2008).

Several observations suggest that follicle cell microvilli play a role in vitelline membrane morphogenesis. First, vitelline bodies form between microvilli (D’Alterio et al., 2005; Kambysellis and Mahowald, 1980). Secondly, in Cad99C mutants, the vitelline bodies were irregular in size, shape and distribution on the apical surface of follicle cells (D’Alterio et al., 2005). In contrast to the palisade mutant, holes in the vitelline membrane of Cad99C mutants persist. Hence, Cad99C mutant females laid eggs that would collapse after deposition due to dessication that is probably caused by these holes. However, the defect seen with the vitelline bodies was probably not due to a defect in protein secretion of Cad99C mutants because when D’Alterio et al. (2005) looked at the distribution of one of the secreted proteins, Nudel, that is involved in proper eggshell formation (LeMosy and Hashimoto, 1998), no difference in Nudel localization was found between Cad99C mutant and wild-type follicles (D’Alterio et al., 2005). How do microvilli mediate and/or control vitelline membrane morphogenesis? One possibility is that microvilli may act as a scaffold for the deposition of vitelline bodies (Schlichting et al., 2006; Elalayli et al., 2008). Therefore, in Cad99C mutants, in which the scaffold is abnormal, vitelline bodies cannot be deposited in a normal pattern. Schlichting et al. (2006) further postulated that the abnormal microvilli resulting from loss of Cad99C do not adequately separate individual vitelline bodies, thus leading to vitelline bodies that are irregular in size, and eventually to a defective vitelline membrane.

As discussed above, defects in microvillus formation affect vitelline membrane formation. Conversely, defects in vitelline membrane morphology also affect microvillus morphology. Recently, the vitelline membrane protein Palisade was shown to influence the pattern of microvilli (Elalayli et al., 2008). However its mechanism of function is unclear. Loss of Palisade at stage 10A, when the microvillus brush border is most prominent in wild-type egg chambers, resulted in disorganized microvilli. In palisade null egg chambers as a result of removal of the palisade gene, apical protrusions were often missing, only few thin processes were seen in these egg chambers. In addition, the perivitelline space between the oocyte and follicle cells was wider compared to wild-type egg chambers. The implication of this is still unclear.
Recent experiments by Elalayli et al. (2008) shed light on how Cad99C might be involved in the formation of the vitelline membrane. Elalayli et al. (2008) first studied the distribution of two major vitelline membrane proteins sV17 and sV23 in Cad99C null egg chambers. In wild-type egg chambers, sV17 and sV23, secreted by the follicle cells, are localized exclusively in the vitelline membrane (Elalayli et al., 2008). In contrast, in Cad99C null egg chambers, while sV23 was properly localized, sV17 was abnormally distributed. The presence of sV17 was reduced at the vitelline membrane, and most of the protein was localized to vesicles in the oocyte cytoplasm (Elalayli et al., 2008). Next, Elalayli et al. (2008) compared the amount of cross-linking sV17 and sV23 in Cad99C null egg chambers. As mentioned previously, vitelline membrane proteins are subjected to proteolytic processing and cross-linking, hence, only reducing agents such as DTT can solubilize them (LeMosy and Hashimoto, 2000; Pascucci et al., 1996;). In stage 14 Cad99C null egg chambers, sV17 and sV23 were stable, as there is no decrease in the amount of the proteins when compared to wild-type egg chambers, shown by Western blotting. Furthermore, sV17 and sV23 were properly processed and cross-linked. In wild-type egg chambers, vitelline membrane proteins undergo further cross-linking (tyrosine) after ovulation (Petri et al., 1976). And by this time, they are completely insoluble even in the presence of DTT (Heifetz et al., 2001). In eggs laid by Cad99C null females, sV17 and sV23 were soluble, indicating that the second cross-linking step was compromised, although there was more cross-linking in sV23 compared to sV17 (Elalayli et al., 2008). To summarize, Cad99C is needed for the stable incorporation of sV17 into the vitelline membrane and in the second cross-linking step of sV23 and sV17 (Elalayli et al., 2008).

5 Objectives

Research in our lab has shown that Cad99C is a regulator of microvillus morphogenesis during Drosophila oogenesis (D’Alterio et al., 2005). To further understand the function of Cad99C as a microvillus regulator, first I conducted experiments to identify molecules that interact with Cad99C and second, I studied their interactions with Cad99C in follicle cell microvilli. As mentioned previously, Pcdh15 was shown to interact with other Usher molecules such as myosin VIIA and Cdh23 (Ahmed et al., 2006; Kazmierczak et al., 2007). Because Cad99C is the orthologue of the mammalian Pcdh15, and has an evolutionarily conserved role in regulating microvillus biogenesis, it may similarly interact with fly homologues of these Usher molecules. To determine whether Cad99C interacts with MyoVIIA, Cad74A and Cad87A, I conducted co-
immunoprecipitation experiments, making use of the available antibodies generated previously in our lab. To study their interactions in follicle cell microvilli, I performed gain-of-function and loss-of-function experiments and analyzed protein distribution using immunostainings. 1) To further characterize the Cad99C-MyoVIIA interaction, I overexpressed MyoVIIA in follicle cells and compared the distribution of Cad99C in wild-type and mutant cells. 2) To characterize the Cad99C-Cad74A interaction, I analyzed the distribution of Cad99C in follicle cells that were either devoid of or ectopically expressed Cad74A and vice versa. 3) To characterize the Cad99C-Cad87A interaction, I analyzed the distribution of Cad99C and Cad87A in follicle cells that overexpressed Cad87A and Cad99C, respectively.

Another objective of my work was to characterize the function of Cad99C, Cad74A, and Cad87A in eggshell morphogenesis. To determine the function of these cadherins and their interacting partners in eggshell morphogenesis, I analyzed the morphology of microvilli and vitelline membrane using Nomarski optics of egg chambers in which Cad99C, Cad74A, and Cad87A were upregulated and/or downregulated, as well as egg chambers that have different ratios of Cad99C and Cad74A, and Cad99C and Cad87A.
# Chapter 2
## Materials and methods

### 1 Drosophila strains

<table>
<thead>
<tr>
<th>Element(s)</th>
<th>Genotype</th>
<th>Description</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cad99C&lt;sup&gt;21z5&lt;/sup&gt;</em></td>
<td><em>w</em>; <em>Cad99C&lt;sup&gt;21z5&lt;/sup&gt;</em></td>
<td>A deletion removing coding sequence for extracellular cadherin domains 1-8 and part of domain 9</td>
<td>D’Alterio <em>et al.</em>, 2005</td>
</tr>
</tbody>
</table>
| • *Cad99C<sup>21z5</sup>*  
  • *Cad99CΔcyt::GFP* | *w*<sup>1</sup>; *Cad99C<sup>21z5</sup>* UAS-*Cad99CΔcyt::GFP* (10) (3rd Chr.) | A *Cad99C* null recombined with UAS-*Cad99CΔcyt::GFP* | This work |
| • *hsFLP1*  
  • *Act5c>CD2>Gal4* | *y*<sup>1</sup>*w*; *hsFLP1*; *Act5c>CD2>Gal4* | **FLP** recombinase under the control of a heat-shock promoter  
• FLP-out cassette that expresses Gal4 gene controlled by Actin5c promoter | Pignoni and Zipursky, 1997 |
| *tj-Gal4* | *y*<sup>1</sup>*w*;*tj-GAL4* (P{GawB}NP1624-5-I) (2nd Chr.) | Enhancer trap line where expression of Gal4 is controlled by the enhancer/promoter of *tj* | Tanentzapf *et al.*, 2007 |
| *UAS-ck::GFP* | *w*<sup>1</sup> UAS-*ck::GFP* (X Chr.) | Full length *ck* fused to GFP under UAS promoter | Todi *et al.*, 2005 |
| • *hsFLP1*  
  • *UAS-mCD8::GFP* | *hsFLP1*, *P{UAS-mCD8::GFP, L}LL4*, *y*<sup>1</sup>*w*; *Pin<sup>56</sup>/CyO* | Mouse transmembrane protein CD8 fused to GFP under UAS promoter | Source: Bloomington  
#5136 |
| *UAS-Cad99C* | *w*<sup>1</sup>; UAS-*Cad99C-FL* (T6) (3rd Chr.) | Full length *Cad99C* under UAS promoter | D’Alterio *et al.*, 2005 |
| *UAS-Cad99C-RNAi* | *w*<sup>1</sup>; UAS-*Cad99C-RNAi* (119) (3rd Chr.)  
*w*<sup>1</sup>; UAS-*Cad99C-RNAi* (106) (3rd Chr.) | • Transgene that induces the expression of *Cad99C* dsRNA resulting in the degradation of *Cad99C* mRNA  
• Under UAS promoter | D’Alterio *et al.*, 2005 |
| *UAS-Cad99CΔcyt::GFP* | *w*<sup>1</sup> UAS-*Cad99CΔcyt::GFP*(18-10) (X Chr.)  
*w*<sup>1</sup>; UAS-*Cad99CΔcyt::GFP*(18-127) (3rd Chr.) | *Cad99C* lacking sequence of cytoplasmic region fused to GFP under UAS promoter | D’Alterio *et al.*, 2005 |
| *UAS-Cad99CΔex::GFP* | *w*<sup>1</sup>; UAS-*Cad99CΔex::GFP*(17M) (3rd Chr.) | *Cad99C* lacking sequence of extracellular region fused to GFP under UAS promoter | Hwang, Liu and Godt, unpublished |
| *UAS-Cad74A::GFP* | *y*<sup>1</sup>*w* UAS-*Cad74A::GFP*(99) (X Chr.)  
*w*<sup>1</sup>; UAS-*Cad74A::GFP*/TM6C (53A) (3rd Chr.) | Full length *Cad74A* fused to GFP under UAS promoter | Lovegrove *et al.*, 2006 |
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<tr>
<th>Transgene</th>
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<tr>
<td>UAS-Cad87A</td>
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<td>Full length Cad87A under UAS promoter</td>
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<td>UAS-Cad74A::GFP (53A)</td>
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<td>Third chromosome with UAS-Cad99C-FL and UAS-Cad74A::GFP</td>
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<tr>
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<td>Third chromosome with UAS-Cad99C-RNAi and UAS-Cad74A::GFP</td>
</tr>
<tr>
<td>UAS-Cad99C-RNAi (119)</td>
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<td>Third chromosome with UAS-Cad99C-RNAi and UAS-Cad74A::GFP</td>
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<tr>
<td>UAS-Cad74A-RNAi</td>
<td>w^1; UAS-Cad99C-FL (T6) UAS-Cad87A-FL (1) (3rd Chr.)</td>
<td>Third chromosome with UAS-Cad99C-FL and UAS-Cad87A-FL</td>
</tr>
</tbody>
</table>

* Recombinant flies were generated by meiotic recombination. Recombinant flies have the darkest orange eye colour due to two copies of the white mini gene in the transgenic constructs. The presence of both transgenic constructs was verified by staining ovaries of recombinant flies with anti-Cad99C and anti-Cad74A/Cad87A antibodies.

## 2 Reagent kits and equipment

- **µMACS GFP Isolation Kit (Miltenyi Biotec):** µMACS Anti-GFP MicroBeads, Wash buffer 1, Wash buffer 2, Elution buffer, µMACS Separator, µ Columns
- **Axioscope 2 fluorescent microscope (Carl Zeiss)**
- **Bicinchoninic acid (BCA) Protein Assay Kit (Pierce)**
- **Biobeads (BioRad)**
- **Centrifuge (Sorvall Legend RT)**
- **Dissecting microscope (Leica MZ6)**
• ECL Western blotting detection reagents (Amersham)
• Hoefer electrophoresis apparatus (SE260B): Mini vertical electrophoresis unit, dual gel caster, constant-current power supply, glass plates, Teflon comb and spacers
• Homogenizer for 1.5 mL eppendorf tubes
• Hyperfilm (Amersham)
• Laser scanning microscope LSM510 (Carl Zeiss)
• Molecular weight markers (10 kDa – 250 kDa, Fermentas #SM1811)
• Nitrocellulose membranes (0.45 µm Schleicher & Schleicher; 0.45 µm GE healthcare)
• Transfer apparatus (GE healthcare): TE62 transfer unit, gel cassettes, foam sponges
• Spectrophotometer (LKB Biochrom Ultrospec 4050)
• Whatman 3MM filter paper
• Vectorshield (Vector Laboratories)

3 Ovary immunostaining

3.1 Dissection

Male and female flies of the right genotype were collected after eclosion in fresh vials. They were fed with ample amount of dry yeast pellets and were left at a 25°C incubator for two days. Both mating and yeast would stimulate oogenesis. After two days, 15-20 female flies were submerged in 95% ethanol in a dissecting dish for two minutes, and were rinsed 3x with 1xPBS (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, pH 7.4). Ovaries were dissected and ovarioles were separated in 1xPBS. After dissection, ovaries were transferred to a 1.5 mL eppendorf tube filled with ice-cold 1xPBS.
3.2 Immunostaining

Ovaries were then fixed with 5% paraformaldehyde or formaldehyde (made fresh each time), in 1 x PBS (pH 7.4) for 10 to 15 minutes. Subsequently, ovaries were rinsed 3x and washed 4 x 15 minutes with PBT (0.3% Triton-X in 1 x PBS pH7.4), incubated with blocking buffer (PBTBS: PBT, 0.1% bovine serum albumin (BSA), 2% goat serum) or ‘blocked’ for 1 hour, and incubated with primary antibodies in PBTBS overnight at 4°C. When anti-Cad99C antibody (GP5) was used as the primary antibody, pre-absorption of the antibodies was done concurrent with the blocking step. GP5 was pre-absorbed with Cad99C-null (Cad99C21-5/Cad99C21-5) mutant ovaries in PBT, with a 1:300 dilution. Refer to Section 3.4 for all primary and secondary antibody concentrations for immunostaining experiments. The next day, after primary antibody incubation, ovaries were rinsed 3x and washed 4 x 15 minutes with PBT. Meanwhile, biobeads (for secondary antibody pre-absorption) were added to new eppendorf tubes. Biobeads were rinsed 3x and washed 4 x 15 minutes with PBT. Although eppendorf tubes were wrapped with aluminum foil to prevent photobleaching of the fluorophores. After washing, ovaries were blocked for 1 hour in PBTBS. Simultaneously, secondary antibodies were added to washed biobeads to pre-absorb for 1 hour. Pre-absorption was done in PBT (1:40 dilution). During and after secondary antibody pre-absorption, eppendorf tubes were wrapped with aluminum foil to prevent photobleaching of the fluorophores. After blocking, ovaries were incubated with pre-absorbed secondary antibodies for 2 hours. Subsequently, ovaries were rinsed 3x and washed 5 x 15 minutes with PBT. Final volume for rinses and washes was 1 mL. Final volume during blocking and pre-absorption, and for primary and secondary antibody incubations was 500 µL. All washes and incubations were done on a nutator. The whole experiment was carried out at room temperature (RT) except for primary antibody incubation, which was done at 4°C. In the case where PB (68.4 mM Na2HPO4, 31.6 mM NaH2PO4, pH 7.2) was used instead of PBS, PB-T (68.4 mM Na2HPO4, 31.6 mM NaH2PO4, 0.3% Triton-X) and PB-TBS (PB-T, 0.1% BSA, 2% goat serum) were used as washing and blocking buffers, respectively. After the final wash, the remaining PBT was pipetted out, and 1:1 PBT/ Vectorshield was added to ovaries to preserve the fluorescence. Ovaries in eppendorf tubes were left at 4°C overnight to rehydrate.
3.3 Mounting and imaging of tissue

The next day, ovaries were mounted in fresh vectorshield and preparations were stored at 4°C. All preparations were observed using a laser scanning microscope (Zeiss LSM510) and all images were analyzed using Zeiss LSM5 Image Browser.

3.4 Antibodies used for immunostaining experiments

<table>
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<th>Primary antibody</th>
<th>Animal immunized</th>
<th>Dilution factor</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Cad99C (pAB RB1) (directed against extracellular cadherin domains)</td>
<td>Rabbit</td>
<td>1:2000</td>
<td>Au Yeung and Godt, unpublished</td>
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<tr>
<td>α-Cad99C (pAB GP5) (directed against extracellular cadherin domains)</td>
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<td>D’Alterio et al., 2005</td>
</tr>
<tr>
<td>α-Cad74A (pAB RB14)</td>
<td>Rabbit</td>
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<td>Kamino and Godt, unpublished</td>
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<tr>
<td>α-Cad87A (pAB GP2)</td>
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<td>Fung et al., 2008</td>
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<tr>
<td>α-Myosin VIIA (pAB GP6)</td>
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<td>Glowinski and Godt, unpublished</td>
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</table>

<table>
<thead>
<tr>
<th>Secondary antibody</th>
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<td>1:400</td>
<td>Jackson Laboratories</td>
</tr>
<tr>
<td>α-rabbit-Cy3</td>
<td>1:400</td>
<td>Jackson Laboratories</td>
</tr>
<tr>
<td>α-guinea pig-Alexa-488</td>
<td>1:400</td>
<td>Molecular Probes</td>
</tr>
<tr>
<td>α-rabbit-Alexa-488</td>
<td>1:400</td>
<td>Molecular Probes</td>
</tr>
</tbody>
</table>
4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

4.1 SDS-PAGE

4.1.1 Tissue preparation

Two-day-old females were collected as described in Section 3. Ovaries (~10 pairs per experiment) were dissected out in ice-cold 1xPBS and were transferred to a 1.5 mL eppendorf tube filled with 2x Laemmli buffer (15 µL of 2x Laemmli buffer per pair of ovaries; 2 x Laemmli buffer: 125 mM Tris-HCl pH6.8, 4.6% SDS, 20% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue) as they were being dissected. Subsequently, ovaries were homogenized thoroughly on ice. After homogenization, the sample was boiled for 5 minutes to further denature the proteins, centrifuged and loaded to a SDS-PAGE gel for subsequent separation (see Electrophoresis below). Alternatively, samples were stored at -80°C.

4.1.2 Length, thickness and percentage of the polyacrylamide gel

Gels were 8 cm long, 9.5 cm wide and 0.75 mm thick. 6% resolving gels and 4% stacking gels were made for all SDS-PAGE experiments. Compositions of the resolving and stacking gels are as follows:

<table>
<thead>
<tr>
<th>Percentage of gel or component</th>
<th>4% (1 stacking gel)</th>
<th>6% (1 resolving gel)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30%:0.8% Acrylamide:Bis-Acrylamide</td>
<td>0.5 mL</td>
<td>1.5 mL</td>
</tr>
<tr>
<td>1.5 M Tris-HCl, pH 8.8</td>
<td>–</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>0.5 M Tris-HCl, pH 6.8</td>
<td>0.5 mL</td>
<td>–</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>80 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>10% (w/v) Ammonium persulfate (APS)</td>
<td>40 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>N,N,N',N'-Tetramethylethylenediamine (TEMED)</td>
<td>4 µL</td>
<td>8 µL</td>
</tr>
<tr>
<td>H₂O</td>
<td>2.8 mL</td>
<td>5.8 mL</td>
</tr>
</tbody>
</table>
4.1.3 Electrophoresis

The electrophoresis unit was assembled and filled with 1 x SDS-PAGE Running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3). Samples were thawed on ice (if they were frozen previously), boiled for 5 minutes, centrifuged at 4000 rpm at RT for 30 seconds and loaded to the gel. For each well, 10-25 µL of sample were loaded, depending on the experiment. Equal volumes of 1x or 2x Laemmli buffer were loaded to empty lanes. For each gel, 5 µL of molecular weight markers (MWM, 10 kDa – 250 kDa) were also loaded to one lane. 20 mA was applied to the unit while the proteins were migrating through the stacking gel. After the proteins had entered the resolving gel, the current was increased to 64 mA. The gel was run until a good separation of the proteins had occurred (evident from the MWMs) and before the bromophenol blue band ran off the gel into the buffer. The amount of time required for electrophoresis was ~2 hours/gel.

4.2 Western blotting

4.2.1 Blotting

Proteins were transferred from the gel to a nitrocellulose membrane (0.45 µm pore size) by applying an electric current. The nitrocellulose membrane was first soaked in water then 1x transfer buffer (25 mM Tris, 192 mM glycine, 0.0375% SDS, 10% methanol, pH 8.3), 5 minutes each. The blotting filter papers and foam sponges were also soaked in 1x transfer buffer briefly. Within the transfer cassette, a sandwich was made (from (-) electrode to (+) electrode): one layer of foam sponge, two pieces of blotting filter paper, gel, nitrocellulose, another two pieces of blotting filter paper and another layer of foam sponge. Before putting the last layer of foam sponge, the sandwich was rolled over gently by a glass pipette to remove air bubbles that would block current flow and consequently, transfer of proteins. The transfer was done at 300 V, 250 mA for 4.5-5 hours, at 4°C. After the transfer, the membrane was either processed immediately (see next section) or left in the transfer unit at 4°C overnight.

4.2.2 Labelling

The membrane or ‘blot’ was washed with ddH₂O briefly for 5 minutes, incubated in 5% blocking buffer (5% nonfat powdered milk (Carnation brand), 0.1% Tween-20) for 1 hour, and incubated with primary antibodies overnight in blocking buffer at 4°C. The purpose of blocking is to allow
non-specific proteins in milk to bind to non-specific binding sites on the nitrocellulose membrane, thereby reducing background. Refer to Section 4.2.5 for primary and secondary antibody concentrations used in Western blotting experiments. When anti-MyoVIIA antibody (GP6) was used as the primary antibody, it was pre-absorbed during the blocking step. GP6 was pre-absorbed with a blot containing cell lysate from ovaries with strongly reduced MyoVIIA expression (10 pairs of $ck^m/ck^m$ ovaries per blot) in PBS-Tween 20 (final volume = 20 mL; 1:400 dilution). For primary antibody incubation, 10 mL of pre-absorbed GP6 was added to 10 mL of 2x blocking buffer. The next day, the blot was rinsed 3x and washed 4 x 15 minutes in washing buffer (0.1% Tween-20 in 1 x PBS (pH7.4)) to remove unbound primary antibodies, incubated with secondary antibodies (conjugated to horseradish peroxidase) in blocking buffer for 1 hour at RT, again rinsed 3x and washed 4 x 15 minutes in washing buffer to wash away unbound secondary antibodies. Final volume for blocking and primary and secondary antibody incubations was 10-15 mL (except when GP6 was used). Volume for washes was 100-200 mL. All washes and incubations were done on an orbital shaker.

4.2.3 Detection

This step was done in the dark room. Equal volumes of ECL detection solutions 1 and 2 were mixed in an eppendorf tube. After the last wash of the membrane, excess buffer was drained from the blot, and then the blot was placed on a piece of Saran wrap with the protein side up. The mixed solution was pipetted onto the blot and allowed to incubate for one minute. Excess ECL solution was drained by touching one corner of the blot against a piece of Kimwipe or paper towel. The blot was placed onto a new piece of Saran wrap and was sealed by folding the wrap over the membrane from both sides. The sealed blot was rolled over gently by a glass pipette to get rid of air bubbles. Next, it was placed in a film cassette with the protein side up. A sheet of autoradiography film was then placed on top of the blot. The cassette was closed and the film was exposed for 30 seconds. The film was then developed immediately. Exposure time was adjusted accordingly depending on the appearance of bands on the first film. For all experiments conducted, it ranged between 1 second to 30 minutes. After the film was developed, the film was aligned with the blot to mark the boundaries and molecular weight markers. Exposure time for each film was also recorded. The blot was stored in fresh washing buffer at 4°C if reprobing of the membrane was needed (see next section).
4.2.4 Stripping and detecting membrane with a different antibody

The membrane was rinsed 3x with washing buffer, incubated in hot stripping buffer (0.1 M Glycine, pH 2.6, 65°C) for 2 x 20 minutes on an orbital shaker. Next, the membrane was rinsed 3x and washed 4 x 15 minutes with washing buffer. The same protocol outlined in Section 4.2.2 was used here for detection with a different antibody.

4.2.5 Antibodies used for Western blotting analyses

<table>
<thead>
<tr>
<th>Primary antibody</th>
<th>Animal immunized</th>
<th>Dilution factor</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Cad99C (pAB RB1)</td>
<td>Rabbit</td>
<td>1:3000</td>
<td>Au Yeung and Godt, unpublished</td>
</tr>
<tr>
<td>α-Cad74A (pAB RB14)</td>
<td>Rabbit</td>
<td>1:10000</td>
<td>Kamino and Godt, unpublished</td>
</tr>
<tr>
<td>α-Cad87A (pAB GP2)</td>
<td>Guinea Pig</td>
<td>1:10000</td>
<td>Fung et al., 2008</td>
</tr>
<tr>
<td>α-GFP (mAB)</td>
<td>Rabbit</td>
<td>1:4000</td>
<td>Clonetech</td>
</tr>
<tr>
<td>α-MyoVIIA (pAB GP6)</td>
<td>Guinea Pig</td>
<td>1:4000</td>
<td>Glowinski and Godt, unpublished</td>
</tr>
<tr>
<td>α-DE-Cad (mAB DCAD1)</td>
<td>Rat</td>
<td>1:100</td>
<td>Gift from T. Uemura; Oda et al., 1994</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary antibody</th>
<th>Dilution factor</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-guinea pig-horseradish peroxidase</td>
<td>1:1500</td>
<td>Jackson Laboratories</td>
</tr>
<tr>
<td>α-rabbit-horseradish peroxidase</td>
<td>1:1500</td>
<td>Jackson Laboratories</td>
</tr>
</tbody>
</table>
5 Immunoprecipitation (IP) and Co-Immunoprecipitation (Co-IP) of GFP-tagged proteins using μMACS GFP Isolation Kit (Miltenyi Biotec)

5.1 Expression of GFP-tagged proteins

To identify interacting partners of MyoVIIA, Cad99C, Cad87A and Cad74A, IP and Co-IP experiments were performed. Expression of transgenic UAS-ck::GFP, UAS-Cad99C∆ex::GFP, UAS-Cad99C∆cyt::GFP (in wild-type or Cad99C mutant background), UAS-Cad74A::GFP, and UAS-CD8::GFP in follicular epithelial cells was driven by tj-Gal4.

5.2 Tissue preparation

Lysis buffer was made and protease inhibitor cocktail was added (Lysis buffer: 50 mM Tris-HCl (pH 8), 150 mM NaCl, 1% NP-40; Protease inhibitor cocktail: 0.3 µM aprotinin, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 µM leupeptin, 1 mM phenylmethanesulphonylfluoride (PMSF)). Female flies were collected as described in Section 3. ~20 flies were killed at a time, and ovaries were dissected in ice-cold 1x PBS. Ovaries were transferred to 300 µL of ice-cold lysis buffer as they were being dissected to minimize protein degradation. After dissection of ~160 pairs of ovaries, they were homogenized thoroughly on ice, centrifuged at 14,000 rpm for 5 minutes at 4°C. The supernatant was transferred to a new eppendorf tube, centrifuged 2x for 5 minutes at 14,000 rpm at 4°C, with the supernatant being transferred to a new eppendorf tube between centrifugation steps. This was to remove most of the cell debris. 10 µL of supernatant were taken out and stored at -80°C for future SDS-PAGE and Western blotting analysis. Final volume of the supernatant was adjusted to 2 mL, and was left on ice until all lines were dissected. Fresh cell lysate was quantified (see below) and used immediately in the subsequent IP experiment.

5.3 Protein quantification using the bicinchoninic acid (BCA) assay

A stock concentration of 2 mg/mL of bovine serum albumin (BSA) was prepared. From the BSA stock, a dilution series was prepared:
<table>
<thead>
<tr>
<th>µg of BSA</th>
<th>0</th>
<th>4</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>µL of stock BSA</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>10</td>
<td>20</td>
<td>40</td>
</tr>
</tbody>
</table>

The final volume was adjusted to 100 µL with ddH₂O. 5 µL of cell lysates with unknown concentration were added to new eppendorf tubes. Next, BCA working reagent was prepared by mixing Reagent A and Reagent B in a 50:1 ratio. 1 mL of BCA working reagent was added to standard and unknown samples. All tubes were incubated at 37°C for 30 minutes. After incubation, all reactions were transferred from eppendorf tubes to cuvettes. Subsequently, absorbance was measured by a spectrophotometer set to 562 nm. A linear regression graph was generated by plotting absorbance readings for each BSA standard on the Y-axis, and µgs of BSA on the X-axis. Lastly, total protein concentration of each unknown sample was calculated from the standard curve. For 160 pairs of ovaries, total amount of protein was measured to be ~6 mg.

### 5.4 IP and Co-IP

50 µL of µMACS anti-GFP Microbeads (Miltenyi Biotec) were added to 2 mL of cell lysate. The µMACS anti-GFP Microbeads are a colloidal suspension of super-paramagnetic Microbeads (50 nm in diameter) conjugated to anti-GFP antibodies. Anti-GFP Microbeads were mixed gently with the cell lysate by pipetting up and down and were allowed to bind to GFP-tagged proteins for 30 minutes on ice. After the magnetic labelling was done, a µ Column was placed in the µMACS Separator (the magnet). Next, the following solutions were pipetted to the column:

1. 200 µL of lysis buffer to equilibrate the column.

2. 2 mL of magnetically labelled cell lysate. Most magnetically labelled GFP-tagged proteins should remain in the column.

3. 4 x 200 µL of Wash buffer 1 (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl pH 8), and 100 µL of Wash buffer 2 (20 mM Tris-HCl pH7.5) to remove unbound molecules.

4. 20 µL of hot Elution buffer (50 mM Tris-HCl pH 6.8, 50 mM dithiothreitol (DTT), 1% SDS, 1 mM EDTA, 0.005% bromophenol blue, 10% glycerol, preheated to 95°C); incubated for 5 minutes.
5. 50 μL of hot Elution buffer to elute the target protein and its interacting partners. μMACS anti-GFP Microbeads remained in the column.

The eluate was aliquoted (12.5 μL/tube) and stored at -80˚C. SDS-PAGE and Western blotting analysis were conducted on the next day.

6 Clonal analysis

6.1 Ectopic expression of transgenes using the FLPout cassette in combination with the Gal4/UAS system

To analyze the effects of loss and overexpression of the atypical cadherins Cad99C, Cad74A, and Cad87A on microvilli and vitelline membrane morphogenesis, mosaic follicular epithelia were generated so that mutant and wild-type cells are side by side. To achieve this, flies carrying UAS-cadherin (either loss-of-function or gain-of-function) were crossed with flies carrying y w hsFLP1; Act5c>CD2>Gal4 to produce progeny of the genotype y w hsFLP1; Act5c>CD2>Gal4 UAS-cadherin. The FLP gene is on the X chromosome, which is under the control of the heat-inducible hsp70 promoter. The FLP catalyzes recombination between homologous target sites termed FLP recombination targets, or FRTs (Golic and Lindquist, 1989; Golic, 1991). On the third chromosome, there are two transgenes: 1) a transgene that consists of the Act5c promoter, followed by the FLPout cassette – the gene CD2 (including a transcriptional terminator, TTS) flanked by two FRT sites, and lastly the Gal4 gene, and 2) the UAS-cadherin transgene. Before heat induced-recombination, Act5c drives the expression of CD2. Gal4 would not be produced because transcription terminates before the second FRT (Fig. 6A). When FLP expression is induced by heat-shock, it catalyzes the recombination between the FRTs, thereby excising out the CD2 gene (Fig. 6B). This places the Act5c promoter directly upstream of Gal4, leading to its transcription activation, and consequently the transcription of the target transgene downstream of UAS (Fig. 5, 6C).

Male and female flies of the right genotype were collected after eclosion in fresh vials. Flies were heat-shocked for 25 minutes in a 37˚C water bath. In an egg chamber, a heat-shock of 25 minutes produces 40-60% of mutant clones in the follicular epithelium. After heat-shock, flies were fed with ample amount of dry yeast pellets and were left at a 25˚C incubator for two days. Ovaries were dissected and immunostainings were done as described in Section 3.
Figure 5 The Gal4/UAS system.

Gal4 is produced in a cell- or tissue-specific pattern specified by the promoter or enhancer. The target gene (Gene X) is fused to the UAS element. When Gal4 is produced, it binds to the UAS element and drives the expression of Gene X in an identical pattern. Modified after Johnston, 2002.
Gal4

Genomic enhancer

Tissue-specific expression of Gal4

Gene X

Tissue-specific expression of X
Figure 6 Ectopic expression of transgenes using the FLPout cassette in combination with the Gal4/UAS system.

(A) Act5c drives the expression CD2. Gal4 is not produced because transcription terminates at TTS, immediately downstream of CD2. (B) After heat-shock induction, FLP mediates the recombination between two cis-acting FRTs, thereby excising out the CD2 gene. (C) Act5c is now directly upstream of Gal4, driving its expression. Gal4 binds to the UAS element, activating Gene X. Cells expressing X are CD2-. Modified after Tabata, 2001.
Approximately 40 clones were analyzed per genotype.

Constructs expressed in the follicular epithelium using this technique include the following:

- $y \text{ w } hsFLP1/UAS-ck::GFP; Act5c>CD2>Gal4/+$
- $y \text{ w } hsFLP1/+; Act5c>CD2>Gal4/UAS-Cad99C-FL$
- $y \text{ w } hsFLP1/+; Act5c>CD2>Gal4/UAS-Cad99C-RNAi$
- $y \text{ w } hsFLP1/+; Act5c>CD2>Gal4/UAS-Cad74::GFP$
- $y \text{ w } hsFLP1/+; Act5c>CD2>Gal4/UAS-Cad74A-RNAi$
- $y \text{ w } hsFLP1/+; Act5c>CD2>Gal4/UAS-Cad87A$
- $y \text{ w } hsFLP1/+; Act5c>CD2>Gal4/UAS-Cad99C-FL UAS-Cad74A::GFP$
- $y \text{ w } hsFLP1/+; Act5c>CD2>Gal4/UAS-Cad99C-RNAi UAS-Cad74A-RNAi$
- $y \text{ w } hsFLP1/+; Act5c>CD2>Gal4/UAS-Cad99C-FL UAS-Cad74A-RNAi$
- $y \text{ w } hsFLP1/+; Act5c>CD2>Gal4/UAS-Cad99C-RNAi UAS-Cad74A::GFP$
- $y \text{ w } hsFLP1/+; Act5c>CD2>Gal4/UAS-Cad99C-FL UAS-Cad87A$

7 Checking for the presence of Cad99C deletion ($\text{Cad99C}^{21-5}$) in double recombinants by PCR

To determine whether the extracellular domains of Cad99C can pull down Cad74A independent of the Cad99C cytoplasmic tail or not, $\text{Cad99C}^{\triangle \text{cyt}}::\text{GFP}$ was overexpressed in a Cad99C-null background. Hence, the recombinant of $w^+; UAS-Cad99C^{\triangle \text{cyt}}::\text{GFP} \text{Cad99C}^{21-5}$ was generated. PCR was performed to check for the presence of $\text{Cad99C}^{21-5}$. 
7.1 Single fly genomic DNA prep

A single male was put into an eppendorf tube. 50 µL of Squishing buffer (10 mM Tris-HCl pH8, 1 mM EDTA, 25 mMNaCl, 200 g/mL Proteinase K) (Proteinase K added fresh) was added to the tube. The fly was then mashed for 10 seconds with a yellow pipette tip. Next, the tube was incubated for 30 minutes at 37˚C. After the incubation, Proteinase K was inactivated at 95˚C for 3 minutes. The tube was briefly centrifuged. The supernatant containing genomic DNA was transferred to a new tube and was subsequently used in the PCR reaction to check for Cad99C. Single fly genomic DNA prep was performed on 16 males.

7.2 PCR reaction

1 µL from the single fly genomic DNA prep was used for each PCR reaction with forward primer CAGTGGATTGCAGTGGATTG and the reverse primer CCGTTGGTTCTCCTTTG. A 2 kb band was expected. Sixteen fly lines were tested for the presence of Cad99C deletion. The composition of the PCR mix is as follows:

<table>
<thead>
<tr>
<th>Components</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x Thermo polymerase buffer (Invitrogen)</td>
<td>1 x</td>
</tr>
<tr>
<td>dNTPs (Amersham)</td>
<td>10 mM</td>
</tr>
<tr>
<td>Forward primer (UPTO-GE21034) CAGTGGATTGCAGTGGATTG</td>
<td>10 µM</td>
</tr>
<tr>
<td>Reverse primer (REVE) CCGTTGGTTCTCCTTTG</td>
<td>10 µM</td>
</tr>
<tr>
<td>Taq polymerase (Invitrogen)</td>
<td>1 U / µL</td>
</tr>
</tbody>
</table>

Total volume per one reaction = 50 µL

The program for the Thermocycler is:

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Initial denaturation</td>
<td>94°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>2. Denaturation</td>
<td>94°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>3. Annealing</td>
<td>58°C</td>
<td>1 minute</td>
</tr>
</tbody>
</table>
4. Extension (go back to step 2 for 30 cycles)  |  72°C  |  2 minutes
5. Final extension  |  72°C  |  5 minutes
6. Stop  |  4°C  |

After the PCR reaction was done, PCR products were loaded to a 0.8% agarose DNA gel for electrophoresis. Only DNA with the $Cad99C^{21-5}$ deletion would be amplified by the primers. DNA without the deletion would not be amplified because the primers would be too far apart. Six recombinants were identified. Line #10 was used for subsequent experiments.
Chapter 3
Results

1 Cad99C-Myosin VIIA interaction

1.1 Overexpressing myosin VIIA caused a redistribution of Cad99C to the basal domain of follicle cell microvilli

Previous research in the lab had shown that when Cad99C is overexpressed in the follicular epithelial cells of the Drosophila ovary, a strongly increased amount of MyoVIIA is recruited to the microvilli and MyoVIIA seems to be distributed throughout the microvilli in contrast to wild-type (Glowinski, M.Sc. Thesis, 2008). To determine the effect of overexpression of MyoVIIA on the distribution of Cad99C, I generated mosaics of MyoVIIA-overexpressing cells in the ovary using the FLPout cassette as a driver.

During stage 10, when MyoVIIA::GFP (full length MyoVIIA tagged with GFP (Todi et al., 2005)) was overexpressed in follicle cells, there was an enrichment of myosin VIIA in the microvilli (Fig. 7 C, F, I), which however appeared restricted to the basal region of microvilli (Fig. 7A, D, G, magenta). Moreover, myosin VII was enriched along the basolateral membranes of follicle cells, as well as throughout the main cell body (Fig. 7C, F, I). Despite the increased amount of MyoVIIA, its distribution was similar to wild type. The distribution of MyoVIIA revealed by the anti-MyoVIIA antibody was the same as the MyoVIIA::GFP distribution (Fig. 7C’, F’, I’), indicating that the anti-MyoVIIA antibody properly reflected where the transgenic protein is expressed.

In MyoVIIA::GFP overexpressing cells, Cad99C was seen enriched in the basal domain of microvilli while its concentration in the apical region seemed reduced (Fig. 7B, E, H). In contrast, Cad99C was distributed evenly throughout the entire length of microvilli in wild-type cells (Fig. 7B, E, H; D’Alterio et al., 2005). In the merged images, MyoVII and Cad99C can be seen to colocalize in the basal region of the microvilli (Fig. 7A, B, C, magenta). In conclusion, expressing myoVIIA::GFP resulted in a relocalization of a considerable amount of Cad99C from the apical domain of microvilli to the basal domain, where myosinVIIA::GFP had accumulated.
Figure 7 Overexpression of MyoVIIA::GFP leads to an enrichment of Cad99C in the basal domain of microvilli.

Confocal cross sections of the follicular epithelium at stage 10A (A-C) and stage 10B (D-I). MyoVIIA::GFP was expressed in follicle cell clones using Act5c>CD2>Gal4. Cad99C is red. MyoVIIA is blue. MyoVIIA::GFP is green. The overlay of Cad99C and MyoVIIA is in magenta. (A-C, D-F, G-I) In cells that expressed MyoVIIA::GFP, Cad99C is enriched in the basal domain of microvilli. In contrast, Cad99C in wild-type cells is uniformly distributed throughout the entire length of microvilli. (C’, F’, I’) The MyoVIIA::GFP distribution is the same as the MyoVIIA distribution revealed by anti-MyoVIIA antibodies. The microvillus brush border is marked by a white arrowhead in A, D and G. White dashed lines mark clone boundaries in all panels. Oc, oocyte; Fc, follicle cells.
Myosin VIIA overexpression

Cad99C MyoVIIA MyoVIIA::GFP
1.2 Cad99C and Myosin VIIA are part of the same protein complex

1.2.1 Myosin VIIA co-immunoprecipitates with the cytoplasmic tail of Cad99C

Previous research showed that 1) The distribution of MyoVIIA and Cad99C overlap in the base of microvilli; 2) Loss of MyoVIIA in follicle cells leads to abnormal microvilli with reduced levels of Cad99C; 3) In Cad99C-deficient follicle cells, apical MyoVIIA is reduced; and 4) Overexpression of Cad99C recruited MyoVIIA from the main body of the cell to the microvilli. However, overexpressing Cad99C that lacks the cytoplasmic region did not lead to the recruitment effect. Interestingly, the cytoplasmic tail of Cad99C alone could recruit cytoplasmic MyoVIIA to the microvilli (Glowinski M.Sc.Thesis 2008). Hence the data point to a possible physical interaction between MyoVIIA and the cytoplasmic region of Cad99C.

To determine whether Cad99C and MyoVIIA are part of the same protein complex or not, Co-immunoprecipitation (Co-IP) experiments were performed. To specifically look for an interaction between the cytoplasmic region of Cad99C and MyoVIIA, I made use of a Cad99C deletion construct in which the entire extracellular region (except the signal peptide) was replaced by GFP, UAS-Cad99C\textasciitilde\textasciitildeGFP (Fig. 8). Functional analysis of Cad99C\textasciitilde\textasciitilde::GFP showed that it acts in a dominant negative manner in affecting follicle cell microvilli (Liu and Godt, unpublished). In stage 10A egg chambers, expression of Cad99C\textasciitilde\textasciitilde::GFP caused a depletion of endogenous Cad99C in the apical plasma membrane of follicular epithelial cells. Furthermore, the microvilli seemed shorter and were abnormal in shape and distribution, reminiscent of those found in Cad99C null follicle cells. Lastly, expressing Cad99C\textasciitilde\textasciitilde::GFP resulted in a large amount of cytoplasmic vesicles that were positive for both endogenous Cad99C and Cad99C\textasciitilde\textasciitilde::GFP, suggesting a high turnover of both proteins. Hence, we are looking for an interaction between MyoVIIA and the cytoplasmic region of Cad99C in a cell environment where microvilli are not normal. Nevertheless, two observations validate the use of the dominant-negative construct: first, Cad99C\textasciitilde\textasciitilde::GFP properly localizes to the short apical microvilli that resulted from Cad99C\textasciitilde\textasciitilde::GFP expression (Liu and Godt, unpublished). Second, as mentioned before, MyoVIIA was enriched in the apical surface of Cad99C\textasciitilde\textasciitilde::GFP-expressing follicle cells (Glowinski, M. Sc. Thesis 2008).
Figure 8 Transgenic proteins used in co-immunoprecipitation and immunostaining experiments.

Schematic showing the structures of untagged and GFP-tagged proteins that were used in co-immunoprecipitation and immunostaining experiments.
To conduct the Co-IP experiment, \textit{UAS-Cad99C\textsubscript{Aex}::GFP} was expressed in follicular epithelial cells using \textit{traffic jam-Gal4 (tj-Gal4)}. Traffic jam is a transcription factor expressed in somatic cells of the \textit{Drosophila} ovary (Li \textit{et al}., 2003). \textit{tj-Gal4} is highly expressed in the follicular epithelial cells during stage 9 to late stage 10B of \textit{Drosophila} oogenesis. Hence using \textit{tj-Gal4} as a driver would result in high expression of the constructs in follicular epithelial cells during these stages. The co-immunoprecipitate from the Co-IP experiment was probed with anti-MyoVIIA antibodies to see whether MyoVIIA co-immunoprecipitates with Cad99C\textsubscript{Aex}::GFP or not.

As a negative control, cell lysate expressing mCD8 fused with GFP was used (Fig. 8). mCD8 is a transmembrane protein from a mouse lymphocyte and is not expected to interact with MyoVIIA (Chen \textit{et al}., 1986; Lee and Luo, 1999). mCD8::GFP, when expressed in the follicular epithelial cells of the \textit{Drosophila} ovary, localizes to all plasma membrane structures including the microvillus brush border (D’Alterio \textit{et al}., 2005). Lastly, lysate from cells expressing MyoVIIA::GFP (full-length MyoVIIA fused with GFP, Fig. 8) was included as a positive control for the anti-MyoVIIA antibody. MyoVIIA::GFP shows the same distribution pattern as endogenous MyoVIIA when expressed in follicular epithelial cells (Fig. 7).

Fig. 9A shows that the cytoplasmic domain of Cad99C pulled down MyoVIIA (Fig. 9A, lane 3). The anti-MyoVIIA antibody detected a protein with an apparent molecular mass of approximately 250 kDa. This corresponds in size to the band that was lost in the myosin VIIA mutant – \textit{ck\textsuperscript{13}} (Kiehart \textit{et al}., 2004). Expectedly, mCD8::GFP did not pull down MyoVIIA, although MyoVIIA was present in the cell lysate before co-immunoprecipitation (Fig. 9A, Lanes 2 and 5). Lastly, transgenic myoVIIA::GFP was properly immunoprecipitated by the anti-GFP antibody (Fig. 9A, Lane 1). Lanes 4 – 6 showed that MyoVIIA was present in the cell lysate before loading through the column, and that equal amounts of cell lysates were loaded (Fig. 9A). At a shorter exposure time, the smear of bands in Fig. 9A was resolved to distinct bands (Fig. 9A’, Lane 1). The upper most band in Lane 1 of Fig. 9A’ is expected to be myosin VIIA::GFP, it migrated to a position slightly higher than the endogenous myosin VIIA molecule.

Myosin VIIA::GFP was predicted to be ~280 kDa, 30 kDa bigger than endogenous myosin VIIA as it is GFP-tagged. Although the detected protein was estimated to be only ~269 kDa, it very likely represents myosinVIIA::GFP because it corresponds to the additional higher band in the raw lysate from cells that overexpressed myosin VIIA::GFP (Fig. 9A’, Lane 4). The additional
**Figure 9 Myosin VIIA interacts with the cytoplasmic tail of Cad99C.**

(A) GFP-tagged proteins were immunoprecipitated from ovarian lysates by magnetically-labeled anti-GFP antibodies. Immunoprecipitates were denatured and separated by SDS-PAGE, and analyzed by Western blotting using polyclonal MyoVIIA antibodies. MyoVIIA co-immunoprecipitated with Cad99CΔex::GFP (Lane 3), whereas it did not with mCD8::GFP (negative control, Lane 2). As a positive control, anti-MyoVIIA antibodies detected transgenic MyoVIIA::GFP and endogenous MyoVIIA (Lanes 1 and 4, after and before immunoprecipitation, respectively). (A’) Film (A) exposed for 1 second to resolve the smear in Lane 1 (Fig. A) to two bands that represented transgenic and endogenous myosin VIIA. Subsequently, the blot in (A-A’) was stripped and reprobed with anti-GFP antibody (shown in B-B’) to verify the presence of GFP-tagged proteins. (B’) Immunoblot in (B) exposed for 2.5 minutes to confirm the presence of MyoVIIA::GFP in the tissue lysate before IP (B’, Lane 4). A protein ladder is indicated by black lines at the left of each blot. Red asterisks indicate proteins of interest.
**A** anti-MyoVIIA (15 sec. film exposure)

IP and Co-IP

lysate

kDa

1 2 3

4 5 6

1, 4: MyoVIIA::GFP

2, 5: mCD8::GFP

3, 6: Cad99CΔex::GFP

**A’** anti-myosin VIIA (1 sec. film exposure)

Co-IP

lysate

kDa

1 2 3

4 5 6

1, 4: MyoVIIA::GFP

2, 5: mCD8::GFP

3, 6: Cad99CΔex::GFP

**B** anti-GFP (5 sec. film exposure)

IP

lysate

kDa

1 2 3

4 5 6

1, 4: MyoVIIA::GFP

2, 5: mCD8::GFP

3, 6: Cad99CΔex::GFP

**B’** anti-GFP (2.5 min. film exposure)

IP

lysate

kDa

1 2 3

4 5 6

1, 4: MyoVIIA::GFP

2, 5: mCD8::GFP

3, 6: Cad99CΔex::GFP
bands below the top most bands in Lanes 1 and 3 in Fig. 9A are either degradation products or other isoforms of MyoVIIA.

To check whether the GFP-tagged proteins were immunoprecipitated by the magnetically labeled anti-GFP antibody, the blot in Fig. 9A was stripped and analyzed with a different anti-GFP antibody that was not magnetically labelled. As it is shown in Fig. 9B, the magnetically labeled GFP antibodies were able to immunoprecipitate the target proteins – ck::GFP, mCD8::GFP, and Cad99CΔex::GFP (Lanes 1, 2, and 3 respectively). The GFP tagged proteins migrated to the expected positions and corresponded to the bands in the cell lysate lanes (Fig. 9B, Lanes 4 – 6). Detection of the ck::GFP signal required a longer exposure time (Fig. 9B’, Lane 4). The size of ck::GFP (~268 kDa) is similar to what was obtained previously (~269 kDa in Fig. 9A’, Lane 1), and the size of Cad99CΔex::GFP (~95 kDa) is in agreement with the predicted ~92 kDa (Fig. 9B, Lanes 3 and 6). mCD8::GFP has a predicted of molecular mass of 54 kDa (Fig. 9B, Lanes 2 and 5). Together, the results further indicate that Cad99C and myosin VIIA are part of the same protein complex.

1.2.2 Testing the specificity of anti-Cad99C antibodies

To further confirm the interaction between Cad99C and myosin VIIA, a second Co-IP experiment was performed by loading cell lysate expressing myosin VIIA::GFP through the column. The resulting eluates were analyzed by an anti-Cad99C antibody. Before the Co-IP experiment was performed, two anti-Cad99C antibodies, RB1 and RB3, were tested in Western blot assays.

RB3 detected a prominent signal in wild-type ovarian tissue lysate at a position corresponding to the 250 kDa protein mass marker (Fig. 10A, Lane 1). This size is bigger than the previously reported value for Cad99C detected by the anti-Cad99C-GP5 antibody (~217 kDa; D’Alterio et al., 2005). However, this protein is likely Cad99C because in an equal amount of lysate from tissue that overexpressed Cad99C, RB3 detected a strongly enhanced signal at the same position (Fig. 10A, Lane 2). RB3 also detected a truncated isoform of Cad99C, which lacks most of its cytoplasmic tail – Cad99CΔcyt::GFP, at approximately the same position as the wild-type and overexpressed Cad99C (Fig. 10A, Lane 3). Cad99CΔcyt::GFP is predicted to have the same molecular mass as full-length Cad99C. These data strongly suggest that RB3 recognizes the Cad99C protein.
Figure 10 Analysis of anti-Cad99C antibodies.

(A) The anti-Cad99C antibody RB3 detected a protein of ~250 kDa in wild-type lysate (Lane 1). A much stronger signal is seen with overexpressing tissue (Lane 2). In lysate of cells that expressed Cad99CΔcyt::GFP, RB3 also gave a strong signal at ~250 kDa. Cad99CΔcyt::GFP is predicted to have a similar molecular mass as full-length Cad99C. (B) The anti-Cad99C antibody RB1 detected a prominent signal close to the 250 kDa marker in lysate that overexpressed full-length Cad99C (Lane 3), but not Cad99C-deficient or wild-type cell lysate (Lanes 1, 2). Equal amounts of tissue lysate were loaded in each lane. A protein ladder is indicated at the left of each blot. Red asterisks indicate proteins of interest.
A anti-Cad99C (RB3)

1: wild-type
2: Cad99C overexpression
3: Cad99CΔcyt::GFP expression

B anti-Cad99C (RB1)

1: Cad99C^{RNAi}
2: wild type
3: Cad99C overexpression
RB1 did not detect a signal for the Cad99C protein in wild-type lysate or lysate that is devoid of Cad99C (Fig. 10B, Lanes 1, 2). However, it recognized a protein of ~250 kDa in lysate that overexpressed full-length Cad99C (Fig. 10B, Lane 3). Moreover, it is similar in size to the protein detected by RB3. Taken together, the immunoblot analysis in Fig. 10 strongly suggest that RB1 and RB3 can properly recognize the Cad99C protein.

1.2.3 Cad99C co-immunoprecipitates with MyoVIIA::GFP

To determine whether Cad99C co-immunoprecipitates with MyoVIIA::GFP or not, RB3 was used to analyze eluates obtained from a Co-IP experiment, in which MyoVIIA::GFP was expressed and immunoprecipitated, followed by washes and elution. Fig. 11A shows that the anti-Cad99C antibody detects a signal in the eluate with an apparent molecular mass of ~250 kDa (Lane 1). This indicates that Cad99C co-immunoprecipitated with MyoVIIA::GFP. mCD8::GFP was again used as a negative control, and no Cad99C was detected in Lane 2 (Fig. 11A). The presence of Cad99C in cell lysate before loading through the column was confirmed in Lanes 3 and 4 (Fig. 11A). To confirm that the GFP-tagged proteins were immunoprecipitated, the blot shown in Fig. 11A was stripped and probed with an anti-GFP antibody (Fig. 11B, B’). As shown in Fig. 11B, GFP-tagged proteins were immunoprecipitated by the magnetically labelled anti-GFP antibody (Fig. 11B, Lanes 1 and 2). They were also present in the cell lysate before loading through the column (Fig. 11B, Lane 4; Fig. 11B’, Lane 3). MyosinVIIA::GFP was estimated to be ~269 kDa, similar to the previously obtained value ~ 268 kDa (Fig. 11B-B’, Lanes 1 and 3). mCD8::GFP migrated to the protein front as expected (Fig. 11B-B’, Lanes 2 and 4).

In conclusion, these Co-IP experiments indicate that MyoVIIA and Cad99C are part of a protein complex in follicular epithelial cells. More specifically, my data show that the Cad99C-MyoVIIA interaction is mediated by the cytoplasmic domain of Cad99C. Whether MyoVIIA can bind directly to Cad99C awaits further analysis (see Discussion).

2 Cad99C-Cad74A interaction

One approach we have taken to further understand the function of Cad99C during microvillus morphogenesis is to identify other Cad99C interactors in follicle cell microvilli. By studying Cad99C interactions with other microvillus molecules, we may gain better insight into how
Figure 11 Cad99C interacts with Myosin VIIA::GFP.

(A) GFP-tagged proteins were immunoprecipitated from ovarian lysate by magnetically-labeled anti-GFP antibodies. Immunoprecipitates were denatured and separated by SDS-PAGE, and analyzed by Western blotting using polyclonal anti-Cad99C antibodies (RB3). Cad99C co-immunoprecipitated with myosin VIIA::GFP (Lane 1) but not mCD8::GFP (negative control, Lane 2). Subsequently, the blot was stripped and reprobed with anti-GFP antibody (shown in B-B’) to verify the presence of GFP-tagged proteins. (B’) Prolonged film exposure confirms the presence of MyoVIIA::GFP in the tissue lysate before IP (Lane 3). A protein ladder is indicated at the left of each blot. Red asterisks indicate the proteins of interest.
**A** anti-Cad99C (RB3)

- **Co-IP**
  - Lanes 1 and 2
  - Marked protein bands

- **lysate**
  - Lanes 3 and 4
  - Marked protein bands

1, 3: MyoVIIA::GFP
2, 4: mCD8::GFP

**B** anti-GFP (30 sec. film exposure)

- **IP**
  - Lanes 1 and 2
  - Marked protein band

- **lysate**
  - Lanes 3 and 4
  - Marked protein band

1, 3: MyoVIIA::GFP
2, 4: mCD8::GFP

**B’** anti-GFP (3min. film exposure)

- **IP**
  - Lanes 1 and 2
  - Marked protein band

- **lysate**
  - Lanes 3 and 4
  - Marked protein band

1, 4: MyoVIIA::GFP
2, 5: mCD8::GFP
microvillus morphogenesis is regulated. Cadherins are known to mediate homophilic as well as heterophilic adhesions (reviewed by Halbleib and Nelson, 2006). As examples of heterophilic adhesion, in mice, N-cadherin and R-cadherin have been shown to bind in a heterophilic manner (Matsunami et al., 1993). And more recently, as discussed earlier, it was shown that the mammalian Cdh23 and Pcdh15 can bind to each other through the N-terminus (Kazmierczak et al., 2007). Because Cad99C and Cad74A are structurally similar to their mammalian homologues Pcdh15 and Cdh23, respectively, there is a possibility that Cad99C and Cad74A may interact via their cadherin domains. During Drosophila oogenesis, Cad74A is first detected at late stage 10B in the columnar follicle cells (Zartman et al., 2008). Specifically, it is enriched in the apical plasma membrane and microvilli (Zartman et al., 2008; Hwang and Godt, unpublished). However, its distribution in the apical membrane is discontinuous and punctate during the initial stage of expression (stage 10B). After stage 10B, Cad74A is expressed at high levels. From stages 11 to 14, it can be seen as a prominent, continuous band that outlines the apical membrane of follicular epithelial cells. Since Cad99C and Cad74A are present in the microvilli from stage 10B onwards, this further raises the possibility that the two molecules may interact in regulating microvillus morphogenesis. Moreover, Cad74A might play a role in vitelline membrane and/or chorion formation since it is strongly expressed during stages (from stage 10B onwards) when the eggshell is being formed.

To look for interactions between Cad99C and Cad74A, and to see the effects of these molecules on microvillus and vitelline membrane morphogenesis, 4 recombinant genotypes were generated, which allowed expression of 1. UAS-Cad99C and UAS-Cad74A::GFP; 2. UAS-Cad99C and UAS-Cad74\textsuperscript{R\textsc{nai}}; 3. UAS-Cad99C\textsuperscript{R\textsc{nai}} and UAS-Cad74A::GFP; and 4. UAS-Cad99C\textsuperscript{R\textsc{nai}} and UAS-Cad74A\textsuperscript{R\textsc{nai}}. Below, I describe the results obtained for each recombinant. Clonal expression of these constructs was induced by the FLPout cassette. For each recombinant, ovaries were stained with anti-Cad99C and anti-Cad74A antibodies to recognize the proteins, except where Cad74::GFP is expressed. GFP expression was strong enough to recognize cells expressing Cad74::GFP. Confocal images were taken to analyze the distribution of the two cadherins. Nomarski images were taken to analyze the effects on the microvillus brush border and the vitelline membrane.
2.1 Overexpression of full-length Cad99C leads to abnormally long microvilli during late stages of oogenesis

To determine whether Cad99C has an effect on microvilli and vitelline membrane morphogenesis and regulation during late oogenesis, I performed gain-of-function analysis for Cad99C and analyzed microvilli and vitelline membrane morphology in Cad99C-overexpressing cells during late stages of oogenesis. During stage 10, when the microvillus brush border is most prominent, overexpression of Cad99C causes an excessive lengthening of microvilli on the apical side of follicle cells (D’Alterio et al., 2005). At this stage, the microvillus brush border forms a distinct band in the Nomarki image (Fig. 4A”; D’Alterio et al., 2005). The stripe pattern reflects vitelline bodies (aggregates of vitelline membrane proteins) alternating with microvilli. At the end of stage 10 and beginning of stage 11, the microvillus brush border begins to regress, concurrent with the start of the fusion of vitelline bodies to form the vitelline membrane. In contrast, Cad99C-overexpressing follicle cells display very long microvilli during these stages (Fig. 12A’, C’). At stages 12 and 13, Cad99C staining did not reveal prominent protrusions seen in earlier stages, although weak Cad99C staining can still be seen on the apical side of Cad99C-overexpressing cells (Fig. 12E’, G’). These data suggest that during stages 12-13, overlong microvilli still form in Cad99C-overexpressing cells, but they are thinner compared to those seen in stages 10-11.

In a wild-type follicle, Nomarski optics revealed a smooth, continuous layer of vitelline membrane that is formed by stage 11 and persists throughout the remainder of oogenesis (Fig 4B”). In Cad99C overexpressing cells, the vitelline membrane did not form a continuous layer during stages 11-13 (Fig. 12B, D, F, H). The vitelline bodies remained separated by microvilli, similar to the pattern seen in wild-type at stage 10 (Fig. 12B, D, F, H). Moreover, Nomarski images showed that in Cad99C overexpressing cells, the depressions that mirror the overlong microvilli were deeper in earlier stages (late 10B to 11) than in later stages (12-13) (compare Fig. 12B, D to Fig. 12F, H). This raises the possibility that, either the size of the overlong microvilli changes over time, thicker in earlier and thinner in later stages, or microvilli of different sizes were formed at different stages.
Figure 12 Overexpression of full-length Cad99C leads to abnormally long microvilli during late stages of oogenesis.

(A-A”, C-C”, E-E”, G-G”) show a confocal cross section of the follicular epithelium. (B, D, F, H) are Nomarski images of (A, C, E, G), respectively. Full length Cad99C was overexpressed in cell clones in the follicular epithelium using Act5c>CD2>Gal4. Cad99C is in red. Cad74A is in green. (A-A’, C-C’, E-E’, G-G’) Overexpressing Cad99C produced overlong microvilli as revealed by Cad99C stainings. The overlong microvilli are more prominent in earlier stages. (B, D, F, H) The long microvilli disrupt the vitelline membrane. (A”, C”, E”, G”) In Cad99C-overexpressing cells, Cad74A localized to the overlong microvilli but the amount of Cad74A in the apical plasma membrane seems reduced. The microvillus brush border is marked by a white arrowhead in A, C, E and G. White dashed lines mark clone boundaries in all panels. Oc, oocyte; Fc, follicle cells. Numbers indicate stages of oogenesis.
To look for an interaction between Cad99C and Cad74A at the cellular level, first I overexpressed full-length Cad99C (Cad99C-FL) and analyzed the distribution of Cad74A in Cad99C-overexpressing cells. As mentioned before, Cad74A is enriched at the apical plasma membrane of follicular epithelial cells from late 10B until the end of oogenesis. In Cad99C-overexpressing cells, Cad74A was seen in the overlong microvilli across all stages (Fig. 12A, C, E, G, A”, C”, E”, G”). Moreover, the amount of Cad74A in the overlong microvilli correlated positively with how much Cad99C was present there. For example, more Cad74A was seen localized along the overlong microvilli at stage 10B when more Cad99C was also seen in the overlong microvilli (Fig. 12A’-A”). At later stages, in Cad99C overexpressing cells, less Cad99C was seen in the apical protrusions, and less Cad74A was similarly observed in the apical protrusions (Fig. 12C’-C”, E’-E”, G’-G”). In addition, for all stages, the apical Cad74A staining in Cad99C overexpressing cells was weaker than that of wild type cells (Fig. 12A”, C”, E”, G”). However, it cannot be concluded that Cad74A was reduced because it was distributed among a larger surface area.

To determine whether the total amount of Cad74A in the apical plasma membrane was affected by Cad99C-overexpression or not, the amount of Cad74A in the apical plasma membrane was compared between wild-type and Cad99C-overexpressing follicle cells. The image analysis program Image J was used to measure anti-Cad74A antibody fluorescence intensities in neighbouring wild-type and mutant follicle cells. Two wild-type and two adjacent mutant cells were evaluated in each confocal image. For each cell, fluorescence was measured in two different areas of the apical membrane (Fig. 13), and the average fluorescence intensity per cell was then calculated. The average fluorescence intensities were then used to calculate 3 ratios: 1) fluorescence intensity from wild-type cell #1 / fluorescence intensity from mutant cell #1, and fluorescence intensity from wild-type cell #2 / fluorescence intensity from mutant cell #2 (w/m ratios; the experiment); 2) fluorescence intensity from mutant cell #1 / fluorescence intensity from mutant cell #2 (m/m; control); 3) fluorescence intensity from wild-type cell #1 / fluorescence intensity from wild-type cell #2 (w/w; control). We would expect that the m/m and w/w ratios would be around one. The number of cases for each fluorescence intensity ratio were counted and plotted in a graph. As shown in Fig. 14, for both stage 10B and 11 or older follicle cells, the w/w and m/m ratios were between 1-1.4. In most cases the ratio was 1.1. In contrast, w/m ratios were more variable. For stage 10B, while a few ratios were lower than 1.5, 11/16
Figure 13 Schematic drawing and confocal image of two mutant and two adjacent wild-type cells, illustrating how anti-Cad74A antibody fluorescence intensities were quantified and compared.

A) For each confocal image, two mutant and two adjacent wild-type follicle cells were evaluated. Fluorescence intensity measurements were taken from the apical side of each cell (red and purple boxes). Two measurements were taken per cell, and an average value for fluorescence intensity was calculated for each cell (M1, M2, W1, and W2). The values were used to calculate three ratios of fluorescence intensity, m/m, w/w, and w/m. (B) A confocal image of wild-type and Cad99C-overexpressing (mutant) follicle cells, showing how the amount of anti-Cad74A antibody fluorescence in the apical side of these cells were measured.
mutant mutant wt wt

\[ m/m = \frac{M1}{M2} \]
\[ w/m = \frac{W1}{M1} \text{ and } \frac{W2}{M2} \text{ or } \frac{W1}{M2} \text{ and } \frac{W2}{M1} \]
\[ w/w = \frac{W1}{W2} \]

mutant cells

wt cells

apical plasma membrane of follicle cells

oocyte

mutant cells

follicle cells

wt cells

10 μm
cases were at or greater than 1.5, indicating that the fluorescence intensity from wild-type cells was at least 1.5 times greater than that of the mutant cells. For stage 11 or older follicle cells, ~50% of the cases also showed a reduction (at least 1.5 times) of fluorescence intensity in mutant cells compared to wild-type cells. These results suggest that overexpressing Cad99C reduces Cad74A in the apical plasma membrane.

2.2 Overexpression of full-length Cad74A leads to disorganized microvilli and a reduction of Cad99C in the microvilli

To determine the effect of gain-of-function of Cad74A on the distribution of Cad99C, and microvilli and vitelline membrane morphology, Cad74A::GFP was overexpressed in follicle cells using the FLPout cassette. Overexpression of Cad74A::GFP resulted in disorganized microvilli in all stages analyzed (Fig. 15A’, E’, G’ for stages 10B, late 10B/11, and 12, respectively). At stage 10B, wild-type microvilli showed a spiky pattern (Fig. 15A, A’). Microvilli within each follicle cell still maintained the fan-shaped profile, with taller microvilli at the centre, and shorter ones at the periphery (Fig. 15A, A’). This pattern was also reflected in Nomarski images independent of Cad99C staining (Fig. 15B). The fan-shaped profiles revealed by Cad99C staining and Nomarski optics, were lost in follicle cell microvilli that overexpressed Cad74A::GFP (Fig. 15A-A’’, B, C-C’’, D). In these cells, microvilli were disorganized, and were projecting in all directions in the extracellular space (Fig. 15A’, C’). In addition, the microvillus brush border appeared taller (Fig. 15C’). In contrast, wild-type microvilli emanating from the apical surface of follicle cells projected straight towards to the oocyte (Fig. 15A`). In the mutant microvilli, Cad99C showed a punctate staining, which was different from wild-type, where the amount of Cad99C was equally distributed along the microvilli (Fig. 15A’, C’). In some cases, the amount of Cad99C seemed reduced, and it was difficult to recognize individual microvilli (Fig. 15A`). From late stage 10B to 12, the microvillus brush border regressed considerably in wild-type (Fig. 15E’, G’), however, individual microvilli were still evident on the apical surface of follicle cells (Fig. 15E’, G’). During these later stages, Cad74A::GFP overexpression resulted in the same disorganized phenotype. Microvilli in mutant follicle cells exhibited a wavy appearance (Fig. 15E-E’`, G-G’`). In addition, the disorganized microvilli appeared to be longer than wild-type microvilli (Fig. 15E’, F, G’, H). Although Cad99C staining appeared to be weaker in the mutant microvilli, it is not certain whether there is a reduction in the total amount of Cad99C protein because Cad99C was distributed over a larger surface area. Similar to earlier
Figure 14 In Cad99C-overexpressing follicle cells, the amount of Cad74A in the apical plasma membrane is measurably reduced.

The control fluorescence intensity ratios, the ratio of Cad74A fluorescence intensity in mutant cell / mutant cell (m/m) and the ratio of Cad74A fluorescence intensity in wild-type cell / wild-type cell (w/w), are between 1-1.5, whereas the ratio of Cad74A fluorescence intensity in wild type cell / mutant cell, w/m, shows a broader distribution. Many w/m ratios are greater than 1.5, suggesting that the amount of Cad74A in the apical plasma membrane of Cad99C-overexpressing cells is reduced.
Anti-Cad74A antibody fluorescence intensity in stage 10B follicle cells

Anti-Cad74A antibody fluorescence intensity in stage 11 or older follicle cells
stage 10B follicles, the apical surface of mutant follicle cells during stages late 10B-12 showed a different pattern in Nomarski images compared to wild-type (Fig. 15F, H). In addition, in some cases, the zone between the oocyte and follicle cells was less distinct compared to wild-type (Fig. 15B, D, H). In all stages analyzed, Cad74A::GFP, when overexpressed, was properly localized to the apical side of follicle cells. Moreover, it colocalized with overlong, disorganized microvilli, as shown by Cad99C staining and in Nomarski images (Fig. 15A, C, E, G). When a different UAS-Cad74A::GFP transgenic line was used to overexpress Cad74 (UAS-Cad74A::GFP #99 was inserted into a different location in the genome), Cad99C was consistently found to be reduced in follicle cells expressing Cad74A::GFP (Fig. 15I’). Similar to the previous observations, the microvilli did not show the fan-shaped profile seen in wild-type cells. However, long and wavy microvilli were not seen here. Instead, Cad99C followed the Cad74A::GFP distribution pattern, which was more confined to the apical membrane rather than projecting into extracellular space (Fig. 15I’’). The difference in phenotype is probably due to differences in the expression level for the two transgenic lines. It is worth to mention that the first Cad74A-overexpression line described was used in all subsequent experiments. Nevertheless, in both cases, mutant microvilli shown in Nomaski images were defective compared to wild-type microvilli. Together, these experiments suggest an antagonistic effect of Cad74A on Cad99C.

2.3 Overexpression of full-length Cad99C and Cad74A leads to a phenotype that shares similarities and differences with Cad99C or Cad74A overexpression alone

As shown above, individual overexpression of Cad99C and Cad74A, respectively, produces very different effects on microvillus and vitelline membrane morphology. To determine whether the Cad99C gain-of-function effect or the Cad74A gain-of-function effect is epistatic, the two cadherins were overexpressed together in the same follicle cells, and microvilli and vitelline membrane morphology was analyzed using Nomarski optics. As shown in Fig. 9, when both cadherins were overexpressed, substantially longer microvilli were produced than in wild type (Fig. 16A’, B, C’, D). However, the long microvilli formed in mutant cells were disorganized, unlike the long microvilli produced when Cad99C was being overexpressed alone. Similar to the microvilli resulting from Cad74A::GFP expression, the microvilli were projecting into all directions, resembling bifurcating tree branches (Fig. 16A’, C’). Still, these microvilli were
Figure 15 Overexpression of full-length Cad74A leads to an overlong and disorganized microvillus brush border, a disrupted vitelline membrane, and a reduction of Cad99C in the microvilli.

Transgenic protein Cad74A::GFP was expressed in cell clones in the follicular epithelium using Act5c>CD2>Gal4. Cad99C is in red. Cad74A::GFP is green. (A-A”, C-C”) In all stages analyzed, before and after the onset of endogenous Cad74A expression at late 10B, follicle cells that expressed Cad74A::GFP displayed overlong and disorganized microvilli, and produced a morphologically abnormal vitelline membrane. (I-I”) In a different UAS-Cad74A::GFP line, cells expressing Cad74A::GFP showed a reduction of Cad99C in the microvilli. (A-A”, C-C”, E-E”, G-G”, I-I”) are confocal images; (B, D, F, H) are Nomarski images of (A, C, E and G), respectively; (A, C, E, and G) are merged images. The microvillus brush border is marked by a white arrowhead in A, C, E and G. White dashed lines mark clone boundaries in all panels. Oc, oocyte; Fc, follicle cells. Numbers indicate stages of oogenesis.
Figure 9

Cad74A::GFP expression

Cad99C  Cad74A::GFP
different from microvilli of cells that only overexpressed Cad74A in that they were thicker, presumably because more Cad99C molecules were available to make microvilli as a result of Cad99C overexpression. Also, Cad74A::GFP, when being overexpressed simultaneously with Cad99C, was localized to the lateral plasma membrane of follicle cells in addition to localizing to the microvilli (Fig. 16A”, C”). Cad99C staining and GFP fluorescence revealed that the transgenic proteins colocalized precisely with each other in the microvilli, as well as in cytoplasmic vesicles and along the lateral membrane (Fig. 16A, C). This raises the possibility of a physical interaction between the two cadherins. To analyze the morphology of microvilli independent of Cad99C, Nomarski images were analyzed. Nomarski images revealed a “tree-bifurcating” phenotype, exactly the same as what was obtained from Cad99C staining (Fig. 16B, B’, D, D’). Also, cells expressing both transgenic proteins do not form a smooth vitelline membrane like that in wild-type cells (Fig. 16B, D). Instead, the vitelline membrane was interrupted by projecting microvilli (Fig. 16B, D). This is also evident in a top view of the apical surface of follicle cells. Mutant follicle cells displayed a carpet-like pattern, characteristic of microvilli. In contrast, wild-type cells exhibited a smooth appearance (Fig. 16E-E’). It is possible that the microvilli protrusions prevent the vitelline bodies from fusing together. The vitelline bodies in cells expressing both Cad99C and Cad74A::GFP were seen as larger blobs compared to those produced when Cad99C was being expressed alone. Also, they were arranged in an irregular fashion, like a bag of marbles, compared to those in Cad99C-overexpressing cells, where they were regularly arranged along the apical side of the cell (compare Fig. 16B, D to Fig. 12B, D). Thus, the pattern in Nomarski images obtained from expressing both Cad99C and Cad74A::GFP was different from the one obtained when expressing Cad74A::GFP alone (compare Fig. 16B, D to Fig. 15B, D). In conclusion, the phenotype resulted from expressing Cad99C and Cad74A was one that showed similarities and differences to the ones obtained when Cad99C and Cad74A were being expressed individually.

2.4 Downregulation of Cad99C leads to a defective microvillus brush border and vitelline membrane and appears to affect Cad74A distribution during late stage 10B – stage 12

Knockdown of Cad99C by RNA interference (RNAi) during stage 10 of oogenesis when Cad99C is expressed maximally resulted in a defective microvillus brush border (D’Alterio et al., 2005; Schlichting et al., 2006). In contrast to wild-type microvilli, Cad99C RNAi resulted in
fewer microvilli or in some cases, microvilli were not detectable. When present, the microvilli were shorter and showed an irregular spiky pattern. Cad99C mutations caused similar microvilli defects, indicating the efficiency of the Cad99C<sup>RNAi</sup> line (D’Alterio et al., 2005). To study microvilli and vitelline membrane morphogenesis in the absence of Cad99C during late stage 10B – stage12 of oogenesis, Cad99C dsRNA was expressed in the follicle cell clones. For all stages analyzed, the vitelline membrane resulting from Cad99C<sup>RNAi</sup> was abnormal compared to wild-type. The resulting vitelline membrane phenotype varied from stage to stage; however, it was consistent for the same stage. Below, the phenotype for each stage is described. In wild-type stage 10B follicle cells, the region between the follicle cells and the oocyte is optically distinct in a Nomarski image (Fig. 17B). This region is where microvilli are found and where eggshell material accumulates. This region will be hereafter referred to as the interface zone. In contrast, the interface zone in mutant cells appears less optically distinct (Fig. 17B). At stage 11, the vitelline membrane in mutant cells appeared to be discontinuous (Fig. 17D, F). Also, at the wild-type-mutant cell clone boundary, the vitelline membrane material of mutant cells appeared to be detached and segregated away from that of wild-type cells (Fig. 17D, F), and gaps are evident in the vitelline membrane (Fig. 17F, arrows). In some cases, the extracellular region apical to the follicle cells seemed broader (Fig. 17F). Lastly, at stage 12, the vitelline membrane in mutant cells appeared different from that of wild-type (Fig. 17H).

To see whether Cad74A distribution was affected in the absence of Cad99C, Cad74A protein distribution was analyzed in follicle cells that expressed Cad99C dsRNA using an anti-Cad74A antibody. Cad74A seemed to be reduced in cells that were devoid of Cad99C at late stage 10B (Fig. 17C”). At stage 11, the effect of Cad99C reduction on Cad74A distribution is variable. Some cells devoid of Cad99C showed a reduction of Cad74A in the apical plasma membrane (Fig. 17C”), while in others, no reduction in the amount of Cad74A was observed (Fig. 17E”). At stage 12, Cad74A showed a broader apical distribution in cells that expressed Cad99C dsRNA (Fig. 17G”). In contrast, Cad74A in wild-type cells showed a prominent narrow band at the apical side (Fig. 17G”). Therefore, a reduction of Cad99C appears to affect Cad74A distribution in a way that is stage dependent.
Figure 16 Overexpression of full-length Cad99C and Cad74A leads to a phenotype that has similarities and differences to the one caused by Cad99C or Cad74A overexpression alone.

Transgenic proteins Cad99C and Cad74A::GFP were expressed in the same cell clones in the follicular epithelium using Act5c>CD2>Gal4. Cad99C is in red. Cad74A::GFP is in green. (A-A’’, C-C’’, E’) show a confocal section of stage 11 egg chambers. (B, D, E) are the respective Nomarski images of (A, C, E). (A-A’’, C-C’’) Overexpressing Cad99C and Cad74A::GFP in the same follicle cells resulted in abnormally long and disorganized microvilli. (B, D) The vitelline membrane above mutant clones is defective. (B’, D’) Cad99C and Cad74A colocalized in the microvilli, along the basolateral plasma membranes of follicle cells, as well as in cytoplasmic vesicles (marked by black arrows). (E-E’) Face on view of the apical side of the follicular epithelium also reveals differences in morphology between wild-type and mutant cells. The microvillus brush border is marked by white arrowhead in A and C. White dashed lines mark clone boundaries in all panels. Oc, oocyte; Fc, follicle cells.
Cad99C and Cad74A overexpression

Cad99C Cad74A::GFP
Figure 17 Downregulation of *Cad99C* leads to defects in the vitelline membrane and affects the distribution of *Cad74A* at late stages of oogenesis.

(A-A”, C-C”, E-E”, G-G”) show a confocal cross section of the follicular epithelium of late stage 10B – stage 12 egg chambers. (B, D, F, H) are the respective Nomarski images. *Cad99C* dsRNA was expressed in cell clones in the follicular epithelium using *Act5c>CD2>Gal4*. *Cad99C* is in red. *Cad74A* is in green. (A, C, E, G) In wild-type cells, *Cad74A* is found at the apical surface of follicle cells. Its distribution partially overlaps with *Cad99C* in the apical microvilli (yellow). (B, D, F, H) Downregulating *Cad99C* by RNAi consistently produced a defective vitelline membrane, although the phenotype varied from stage to stage. (A”, C”, E”, G”). The amount of *Cad74A* appeared to be reduced in mutant cells during late stage 10B/11 (A”, C”) but was comparable to wild type during stages 11-12 (E”, G”). The microvillus brush border is marked by a white arrowhead in A, C, E and G. White dashed lines mark clone boundaries in all panels. Black arrows in (F) point to gaps in the vitelline membrane of mutant cells. Oc, oocyte; Fc, follicle cells. Numbers indicate stages of oogenesis.
*Cad99C downregulation by Cad99C<sub>RNAi</sub>*

**A**

**A’**

**B**

**A”**

**C**

**C’**

**D**

**C”**

**E**

**E’**

**F**

**E”**

**G**

**G’**

**H**

**G”**
2.5 Cad74A is not essential for microvillus brush border and vitelline membrane formation or Cad99C localization to the microvilli

To study vitelline membrane morphogenesis in the absence of Cad74A, Nomarski optics was used to analyze vitelline membrane of follicle cells that were devoid of Cad74A. Cad74A was downregulated by expressing UAS-Cad74A\textsuperscript{RNaI} (VDRC 36320). In follicle cells that express UAS-Cad74A\textsuperscript{RNaI} (VDRC 36320), the amount of Cad74A was barely detectable, indicating the efficiency of the RNAi line (Zartman et al., 2008). Downregulating Cad74A resulted in a vitelline membrane that was very similar to the wild-type vitelline membrane at late stage 10B and onwards (Fig. 18B and D for stages late 10B and 11, respectively), and no discernable difference was detected between wild-type and mutant vitelline membranes.

To see whether Cad99C distribution was affected by the loss of Cad74A, anti-Cad99C antibody was used to reveal the Cad99C localization pattern. Fig. 18 A’ and C’ show that Cad99C localization was unaffected in cells that expressed UAS-Cad74A\textsuperscript{RNaI}. In both wild-type and mutant cells, Cad99C was found in the apical microvilli of follicle cells. However, the amount of apical Cad99C in mutant cells appears to be reduced, though quantification is needed to validate this observation.

Together, these results suggest that during late stage 10B to stage 12, microvilli and vitelline membrane form properly in the absence of detectable Cad74A. In other words, Cad74A appears not to be essential for microvillus and vitelline membrane formation during these stages.

It is worthwhile to mention that in wild-type follicle cells, the apical distribution of Cad74A changes in relation to Cad99C as oogenesis proceeds. At late stages 10B, Cad74A is localized more basal to Cad99C (Fig. 18A). At stage 11, Cad74A shows a broader distribution and is more apical than Cad99C (Fig. 18C).
Figure 18 Cad74A is not essential for microvillus brush border/vitelline membrane formation or Cad99C localization to the microvilli.

(A-A”, C-C”) show a confocal cross section of the follicular epithelium of egg chambers. (B, D) are Normarski images of (A-A”, C-C”), respectively. (A-B) is a late stage 10B egg chamber. (C-D) is a stage 11 egg chamber. Expression of Cad74A dsRNA in follicle cell clones was induced by Act5c>CD2>Gal4. Cad99C is in red. Cad74A is in green. (A-A”, C-C”) Downregulating Cad74A in follicle cells during late stage 10B – stage 11 had no effect on Cad99C distribution. (B, D) Expressing Cad74A dsRNA produced no detectable effect on microvillus/vitelline membrane morphogenesis. The microvillus brush border is marked by a white arrowhead in A and C. White dashed lines mark clone boundaries in all panels. Oc, oocyte; Fc, follicle cells.
Cad74A downregulation by Cad74A^RNAi

Cad99C  Cad74A
2.6 Combined reduction of Cad99C and Cad74A caused defects in the vitelline membrane similar to those induced by loss of Cad99C alone

To study microvillus and vitelline membrane morphogenesis in the absence of both Cad99C and Cad74A, a recombinant fly line was generated that contains both \textit{UAS-Cad99CRNAi} and \textit{UAS-Cad74A\textsuperscript{RNAi}} elements. During late stage 10B to stage 12 of oogenesis, Cad99C and Cad74A colocalize at the short apical microvilli (Fig. 19A"’, C"’, E"’, G"’). Nomarski images of cells devoid of both Cad99C and Cad74A, revealed a microvillus brush border and a vitelline membrane that were different than those in wild-type cells (Fig. 19B, D, F, H). In addition, the phenotypic characteristics resembled the ones produced by the loss of Cad99C. In double mutant follicle cells, the interface zone between the oocyte and follicle cells seemed expanded (Fig. 19B and H similar to Fig. 17B and H, respectively). In addition, it is optically less distinct from the oocyte and follicle cells compared to that of wild-type cells (Fig. 19D and F similar to Fig. 17D and F, respectively). In conclusion, the vitelline membrane phenotype that resulted from the loss of both, Cad99C and Cad74A showed striking similarity to the one that resulted from the loss of Cad99C alone, further supporting that Cad74A is not essential for the formation of vitelline membrane during late stages of oogenesis.

2.7 Overexpression of Cad99C can produce overlong microvilli in the absence of Cad74A, resulting in a disrupted vitelline membrane

Because \textit{Cad99C} expression decreases after stage 10B, other cadherins such as Cad74A that are expressed strongly in the apical microvilli during late stages of oogenesis might be involved in regulating microvillus and vitelline membrane morphogenesis. To see whether Cad99C can promote microvillus growth during stages 11-12 of oogenesis in the of specifically Cad74A, a recombinant fly line was generated by meiotic recombination containing \textit{UAS-Cad99C} and \textit{UAS-Cad74A\textsuperscript{RNAi}}. As shown in Fig. 14, overexpression of Cad99C and loss of Cad74A in the same follicle cells led to a stronger outgrowth of microvilli than in wild-type cells, at stages 11 (Fig. 20A"’”) and 12 (Fig. 20C-C””). At stage 11, as revealed by Cad99C staining, overexpressing Cad99C in Cad74A-deficient cells resulted in regularly arranged overlong microvilli protruding from the apical surface of follicle cells (Fig. 20A’). The phenotype was similar to the one in cells that overexpressed Cad99C but were wild type for
Figure 19 Combined reduction of Cad99C and Cad74A caused defects in the vitelline membrane similar to those induced by loss of Cad99C alone.

(A-A”, C-C”, E-E”, G-G”) show a confocal cross section of the follicular epithelium of egg chambers. (B, D, F, H) are the respective Nomarski images. Expression of Cad99C dsRNA and Cad74A dsRNA in the same follicle cell clones was induced by Act5c>CD2>Gal4. Cad99C is in red. Cad74A is in green. (A-H) In cells that are devoid of both Cad99C and Cad74A, the microvillus brush border and vitelline membrane are defective. Gaps are evident in the vitelline membrane at the boundary between wild-type and mutant cells (black arrows). The microvillus brush border is marked by a white arrowhead in A, C, E and G. White dashed lines mark clone boundaries in all panels. Oc, oocyte; Fc, follicle cells. Numbers indicate stages of oogenesis.
Cad99C and Cad74A downregulation
Cad74A (Fig. 12A-A", B). A Nomarki image shows that vitelline bodies are still segregated by the overlong microvilli in mutant cells, whereas a smooth fused vitelline membrane is evident in wild type cells. At stage 12, although the microvilli in mutant cells were shorter and thinner compared to those at stage 11 (Fig. 20C-C"), nevertheless the mutant microvilli resembled the microvilli produced when Cad99C was overexpressed in a wild-type background (Fig. 12E-E", F). Similar to stage 11, the vitelline membrane was interrupted by the protruding microvilli, which can be seen both in a side (Fig. 20C, D) and a top view (Fig. 20E-E’). This experiment suggests that at later stages (stage 11-12), Cad99C is sufficient to produce or maintain overlong microvilli in the absence of Cad74A. In other words, Cad74A is not required to sustain the microvilli at later stages.

2.8 In the absence of Cad99C, Cad74A overexpression cannot generate microvilli

As shown in Fig. 15, when Cad74A::GFP was overexpressed in a wild-type background, long and wavy microvilli were produced. To determine whether Cad74A can produce defects in the absence of Cad99C, a recombinant containing \textit{UAS-Cad74A::GFP} and \textit{UAS-Cad99C}\textsuperscript{RNAi} was generated. Analysis of this recombinant at stage 10B showed that when Cad74::GFP was expressed in cells that were Cad99C-deficient, only shortened protrusions can be seen (Fig. 21A-B). Cad74A::GFP remained at the apical plasma membrane. Expressing Cad74A::GFP in the absence of Cad99C during late stage 10B and 11 produced the same phenotype (Fig. 21C-F). Figs. 21D and F show that, at late stage 10B and stage 11, the vitelline membrane on the apical side of wild-type cells is defective, resembling the \textit{Cad99C}\textsuperscript{RNAi} phenotype shown in Fig. 17B. These observations are consistent with the model that Cad99C is needed for microvillus outgrowth (D’Alterio et al., 2005; Schlichting et al., 2006). Cad74A by itself appears not to be sufficient to form a microvillus brush border.

2.9 Cad99C and Cad74A are part of the same protein complex

Several pieces of data suggest an interaction between Cad99C and Cad74A. First, as mentioned before, the mammalian homologues of Cad99C and Cad74A, Pcdh15 and Cdh23, respectively, physically bind to each other (Kazmierczak et al., 2007). Second, both cadherins associate with follicle cell microvilli (Zartman et al., 2008; Hwang and Godt, unpublished). And lastly, when both proteins are overexpressed simultaneously in follicle cells, they colocalized precisely in the
Figure 20 Overexpression of Cad99C can promote microvillus growth in the absence of Cad74A, resulting in a disrupted vitelline membrane.

(A’-A”, C’-C”, E’) show a confocal cross section of the follicular epithelium. (B, D, E) are the respective Nomarski images. (A, C) are merged imaged of (A’-A”, B) and (C’-C”, D), respectively. (A-B) is a stage 11 egg chamber. (C-E) is a stage 12 egg chamber. Transgenic Cad99C and Cad74A dsRNA were expressed in the follicle cell clones using Act5c>CD2>Gal4. Cad99C is in red. Cad74A is in green. (A-A”, C-C”) Side view of the follicular epithelium showing that during stages 11-12, Cad99C overexpression promotes excessive microvillus elongation even in the absence of Cad74A. (B, D) The overlong microvilli produced as a result of Cad99C overexpression disrupt the smooth vitelline membrane. (E-E’) Face on view of the interface between follicle cells and oocyte showing that at stage 12, long microvilli disrupt the vitelline membrane layer that is evident in wild-type cells. The microvillus brush border is marked by a white arrowhead in A and C. White dashed lines mark clone boundaries in all panels. Oc, oocyte; Fc, follicle cells.
Cad99C overexpression and Cad74A downregulation
Figure 21 In the absence of Cad99C, Cad74A overexpression cannot generate elongated microvilli.

(A’-A”, C’-C”, E’-E”) show a confocal cross section of the follicular epithelium. (B, D, F) are the respective Nomarski images. (A, C, E) are the merged images of (A’-A”, B), (C’-C”, D) and (E’-E”, F), respectively. Cad99C dsRNA and transgenic protein Cad74A::GFP were co-expressed in follicle cell clones using Act5c>CD2>Gal4. Cad99C is in red. Cad74A::GFP is green. (A-A”, B) At stage 10B and later, only small irregular protrusions are seen on the apical surface of cells that express Cad74A::GFP but lack detectable amounts of Cad99C. The stripe pattern characteristic of wild-type cells is lost in mutant cells. (C-C”, D, E-E”, F) During late stage 10B – stage 11, the vitelline membrane above Cad99C-deficient and Cad74A::GFP-expressing follicle cells appears abnormal in morphology or missing. (A”, C”, E”) In the absence of Cad99C, Cad74A::GFP remained on the apical surface of follicle cells. The microvillus brush border is marked by a white arrowhead in A, C and E. White dashed lines mark clone boundaries in all panels. Oc, oocyte; Fc, follicle cells. Numbers indicate stages of oogenesis.
Cad99C downregulation and Cad74A::GFP expression
microvilli, cytoplasmic vesicles and along the lateral plasma membrane (Fig. 16). Co-
immunoprecipitation experiments were performed to see whether Cad99C and Cad74A
molecularly interact. Before co-immunoprecipitation experiments were conducted, I tested our
anti-Cad74A antibodies in a Western blot assay. Ovarian tissue lysates of 3 different genotypes
were analyzed: wild-type, tj-Gal4; UAS-Cad74A::GFP (tissue lysate expressing full length
Cad74A tagged with GFP, Fig. 8), and tj-Gal4; UAS-Cad74A RNAi (tissue lysate expressing ds
Cad74A RNA). Cad74A is predicted to be a 201 kDa protein from its amino acid sequence.
However, in wild-type tissue lysate, anti-Cad74A antibodies prominently detected a protein with
a size of ~262 kDa (Fig. 22D, Lane 3). It is possible that Cad74A, being a membrane protein,
undergoes glycosylation, thus resulting in a bigger protein. In tissue lysate that expressed
Cad74A dsRNA, the band detected in wild type was absent (Fig. 22D, Lane 1), showing that the
detected protein is Cad74A, and confirming the specificity of the antibody. Anti-Cad74A
antibodies produced strong signals for tissue lysate that expressed Cad74A::GFP (Fig. 22D, Lane
2). The upper most band is expected to be Cad74::GFP. To better resolve the protein bands that
correspond to Cad74A::GFP and endogenous Cad74A, respectively, a second immunoblot was
carried out (Fig. 22E), in which Cad74A::GFP was estimated to be ~279 kDa and endogenous
Cad74A was ~260 kDa (Fig. 22E, Lanes 1 and 2, respectively). Cad74A::GFP is ~ 19 kDa
bigger than endogenous Cad74A, close to the expected difference in mass due to the GFP tag
(~30 kDa). In conclusion, the anti-Cad74A antibody properly recognized the Cad74A protein.
Hence, it was used in subsequent immunoprecipitation experiments to identify the Cad74A
protein in eluates.

To determine whether a molecular interaction exists between Cad99C and Cad74A, a co-
immunoprecipitation experiment was conducted using cell lysate expressing an isoform of
Cad99C that lacks the cytoplasmic domain of Cad99C (Cad99C∆cyt::GFP; Fig. 8). tj-Gal4 was
used to drive Cad99C∆cyt::GFP expression in the follicular epithelial cells. Cad99C∆cyt::GFP
and its interacting partner(s) (if any) were immunoprecipitated using magnetically labelled anti-
GFP antibodies. After loading cell lysate + anti-GFP antibody through the column, collected
eluate was probed with the anti-Cad74A antibody. The resulting immunoblot shows that
Cad99C∆cyt::GFP was able to pull down Cad74A (Fig. 22A, Lane 1). The protein band was
estimated to be ~263 kDa, similar to previously obtained values (~262 kDa and ~260 kDa in
Figs. 22D and E, respectively). Cad74A is barely detectable in the negative control lane
Figure 22 Cad74A interacts with the extracellular region of Cad99C, independent of the cytoplasmic tail of Cad99C.

(A) GFP-tagged proteins were immunoprecipitated from ovarian lysates by magnetically-labeled anti-GFP antibodies. Cad74A was detected with anti-Cad74A RB14 antibodies. Cad74A co-immunoprecipitated with Cad99CΔcyt::GFP that was either expressed in a wild-type (Lane 1) or a Cad99C21-5 mutant background (Lane 3). (B) Blot (A) was stripped and probed with anti-GFP antibodies to indicate the presence of GFP-tagged proteins before and after the IP. (C) Eluate from the Co-IP experiment in (A) was analyzed with anti-DE-Cad antibodies. DE-Cad did not co-immunoprecipitate with Cad99CΔcyt::GFP. (D) The anti-Cad74A antibody RB14 was used to analyze Cad74A expression in wild-type, Cad74A-knock down and Cad74A-overexpression ovarian tissue lysates. Equal amounts of tissue lysate were loaded in each lane. RB14 detects a protein in wild-type lysate (Lane 3) that is missing in Cad74A-knock down lysate (Lane 1) but is very prominent in lysate that expressed Cad74A::GFP (Lane 2). (E) Wild-type and Cad74A::GFP-expressing tissue lysates were analyzed by RB14. As expected, in lysate from tissue that expressed Cad74A::GFP, RB14 detected both transgenic Cad74A::GFP and endogenous Cad74A. Protein ladders are indicated by black solid lines at the left of each blot. Red asterisks indicate proteins of interest.
A anti-Cad74A

Co-IP

lysate

kDa 1 2 3

~263 250

1, 4: Cad99CΔcyt::GFP
2, 5: mCD8::GFP
3, 6: Cad99CΔcyt::GFP & no endogenous Cad99C

B anti-GFP

IP

lysate

kDa 1 2 3

~260 250

1, 4: Cad99CΔcyt::GFP
2, 5: mCD8::GFP
3, 6: Cad99CΔcyt::GFP & no endogenous Cad99C

C anti-DE-Cad

Co-IP

lysate

kDa 1 2 3 4

~159 130

mCD8::GFP
Cad99CΔcyt::GFP
mCD8::GFP & no endogenous Cad99C
Cad99CΔcyt::GFP & no endogenous Cad99C

D anti-Cad74

kDa 1 2 3

~262 250

E anti-Cad74

kDa 1 2

~279 260

Cad74A
Cad74A::GFP
Cad74A
Cad74A::GFP
(mCD8::GFP; Fig. 22A, Lane 2). This very faint band is probably due to an incomplete wash of the column. This result suggests that the extracellular cadherin domains of Cad99C are responsible for the interaction. However, because endogenous Cad99C was available in the cell lysate, it is possible that the cytoplasmic domain of Cad99C could participate in mediating the interaction. To rule out this possibility, cell lysate expressing Cad99CΔcyt::GFP in a Cad99C null mutant background was used for a co-immunoprecipitation experiment. Lane 3 in Fig. 22A confirms that the Cad99C-Cad74A interaction was mediated via the extracellular cadherin domains of Cad99C, independent of the cytoplasmic tail. As controls for the co-immunoprecipitation experiment, I confirmed the presence of Cad74A in the raw cell lysates (Fig. 22A, Lanes 4-6). Furthermore, an aliquot of the eluate from the co-immunoprecipitation experiment was analyzed by Western blot using an anti-GFP antibody to verify that the GFP-tagged proteins were immunoprecipitated by the magnetically-labelled anti-GFP antibodies. Anti-GFP antibodies identified Cad99CΔcyt::GFP to be ~250 kDa in both wild-type and Cad99C-null tissues (Fig. 22B, Lanes 1 and 3), similar in size to full length Cad99C as expected. mCD8::GFP migrated to the protein front (Fig. 22B, Lane 2). The GFP-tagged proteins were also identified in cell lysate before immunoprecipitation (Fig. 22B, Lanes 4-6). They migrated to their expected positions.

To further evaluate whether the interaction between the cadherin domains of Cad99C and Cad74A is specific, I tested whether the cadherin domains of Cad99C could similarly interact with another cadherin molecule. The co-immunoprecipitation eluate from the experiment described in Fig. 22A Lane 3 was analyzed with an anti-DE-Cad antibody. Drosophila E-Cadherin (DE-Cad) is a classical cadherin encompassing 7 extracellular cadherin domains (Oda et al., 1994). In the follicular epithelium of the Drosophila egg chamber, it is localized to the zonula adherens in the apico-lateral surface of epithelial cells (Niewiadomska et al., 1999). Fig. 22C shows that DE-Cad did not co-immunoprecipitate with the cadherin domains of Cad99C, but was present in the cell lysate before immunoprecipitation. The anti-DE-Cad antibody detected a band that was ~159 kDa, which is close to the published value of 150 kDa (Oda et al., 1994). In conclusion, these results suggest that the extracellular cadherin domains of Cad99C interact specifically with Cad74A. In other words, it was not an interaction between any two cadherins.
To further confirm the interaction between the extracellular cadherin domains of Cad99C and Cad74A, the reverse experiment was done by immunoprecipitating Cad74A::GFP from cell lysate with the magnetically-labelled anti-GFP antibody, and probing the immunoprecipitate with an anti-Cad99C antibody. As shown in Lane 1 of Fig. 23A, instead of a single band detected by the anti-Cad99C antibody as in Fig. 10B, three bands were detected. The lowest band corresponds in size to the Cad99C signal seen in previous blots (~250 kDa; Fig. 10B; Fig. 23A, Lanes 3 and 4). The two additional bands were calculated to be ~271 kDa and ~282 kDa. They were reproducibly co-immunoprecipitated with Cad74A::GFP in three independent experiments. These two additional bands could be other glycosylated forms of Cad99C. Moreover, because these isoforms of Cad99C were enriched in the Cad74A::GFP immunoprecipitate, and were not detected in the raw cell lysates, this suggests that Cad74A primarily interacts with these isoforms of Cad99C. Also, these modified forms of Cad99C that interact with Cad74A constitute only a minor fraction of total amount of Cad99C, as they were not detected in the cell lysates before co-immunoprecipitation. The blot was stripped to show that Cad74::GFP and mCD8::GFP were immunoprecipitated by the magnetically-labelled antibodies (Fig. 23B). Cad74A::GFP was estimated to be ~281 kDa (Fig. 23B, Lanes 1 and 3), similar to the previous value ~279 kDa (Fig. 22 E).

3 Cad99C-Cad87A interaction

Another molecule possibly interacting with Cad99C and having a role in vitelline membrane mophogenesis and eggshell formation is Cad87A. As described previously, Cad87A is expressed in the follicular epithelial cells and its distribution overlaps with that of Cad99C in the apical microvilli. It is strongly expressed in follicle cells from stage 10B onwards. Because Cad87A partially colocalizes with Cad99C, and is strongly expressed during stages when the eggshell layers are being formed, it may play a role in microvillus and vitelline membrane morphogenesis. A gain-of-function analysis was undertaken to study the function(s) of Cad87A and Cad87A in combination with Cad99C in microvillus and vitelline membrane morphogenesis. Cad87A is a homologue of Drosophila Cad74A mammalian Cdh23. And because Cad99C interacts with Cad74A as shown before, Cad87A could also be in the same protein complex as Cad99C.
Figure 23 Cad99C interacts with Cad74A::GFP.

(A) GFP-tagged proteins were immunoprecipitated from ovarian lysates by magnetically-labeled anti-GFP antibodies. Cad99C was detected with anti-Cad99C-RB1 antibodies. Three protein bands are seen in Lane 1, in which Cad74A::GFP-expressing tissue lysate was used in the experiment, showing that Cad99C co-immunoprecipitated with Cad74A::GFP. However, two protein bands ran higher than the wild-type Cad99C protein band as seen in Lanes 3 and 4, the raw cell lysate lanes. A weak signal was also detected in the negative control lane (Lane 2). In addition, the top two protein bands seen in Lane 1 are missing in Lane 2. (B) Blot (A) was stripped and probed with anti-GFP antibodies to show the presence of GFP-tagged proteins before and after immunoprecipitation. Protein ladders are indicated by black solid lines at the left of each blot. Red asterisks indicate proteins of interest.
A anti-Cad99C (RB1)

Co-IP lysate

1 2 3 4

kDa ~282 ~271 250

~281 kDa

B anti-GFP

IP lysate

1 2 3 4

kDa ~281 250

~281 250 kDa

mCD8::GFP Cad74A::GFP mCD8::GFP Cad74A::GFP

mCD8::GFP Cad74A::GFP mCD8::GFP Cad74A::GFP
3.1 Overexpression of Cad99C leads to a reduction of Cad87A

To study the Cad99C-Cad87A interaction, Cad99C was overexpressed in follicle cells clonally using the FLPout cassette, and changes in Cad87A distribution were analyzed in follicle cells from late stage 10B to stage 12. As shown in Figs. 26A’-A” and B-B”, at late stage 10B, when Cad87A expression at the apical side of follicle cells is still not as prominent, overexpressing Cad99C resulted in a reduction of Cad87A at the apical side of follicle cells. The same phenotype was observed in stages 11 and 12, when Cad87A is strongly enriched in the apical region of follicle cells (Fig. 24C”, D-D”). Figs. 24D-D” shows that Cad87A colocalized with Cad99C in the microvilli at stage 12 of oogenesis. The effect of gain-of-function of Cad99C on microvillus and vitelline membrane morphogenesis was described in Section 2.1.

To determine whether overexpressing Cad99C results in a reduction of Cad87A at the apical plasma membrane or not, the amount of Cad87A was compared between wild-type cells and cells that overexpressed Cad99C. A quantitative analysis similar as described in Section 2.1 was carried out. As shown in Fig. 25, the control ratios, Cad87A fluorescence intensity in mutant cell / mutant cell (m/m) and Cad87A fluorescence intensity in wild-type cell / wild-type cell (w/w) were mostly between 1-1.5. In contrast, the values for Cad87A fluorescence intensity in wild-type cell / mutant cell (w/m) were more spread out. Many cases showed a reduction of Cad87A when Cad99C was overexpressed. Together, these results suggest that Cad99C overexpression affects Cad87A localization at the apical plasma membrane.

3.2 Overexpression of Cad87A may cause defects in microvilli

To further study the Cad99C-Cad87A interaction, Cad87A was overexpressed in follicle cells. Subsequently Cad87A and Cad99C protein distributions were analyzed by Cad87A and Cad99C stainings, respectively. Lastly, microvilli and vitelline membrane morphology were studied by Nomarski optics. At stage 10B, Cad87A was localized along the apical and lateral membranes of Cad87A-overexpressing cells (Fig. 26A’, B’, C’). On the apical side, Cad87A was localized to the microvilli (Fig. 26A, B, C). In addition, Cad87A was seen in the cytoplasm as well as localized to cytoplasmic vesicles (Fig. 26A’, B’ C’). At stage 11, overexpression of Cad87A resulted in the same phenotype as that of late stage 10B (Fig. 26D-D”). However, Cad87A was no longer found along the lateral membranes of follicle cells (Fig. 26D”). Anti-Cad87A antibodies did not stain endogenous Cad87A in wild-type cells possibly because they were
Figure 24 Overexpression of Cad99C may lead to a reduction of Cad87A during late stages of oogenesis.

All panels show a confocal cross section of the follicular epithelium. Transgenic protein Cad99C was expressed in follicle cell clones using Act5c>CD2>Gal4. Cad99C is in red. Cad87A is in green. (A-A”, B-B”, C-C”, D-D”) During late stage 10B to stage 12 of oogenesis, Cad99C overexpression caused a reduction of Cad87A in the apical surface of follicle cells compared to wild-type cells. Consistent with previous experiments, overexpressing Cad99C produced overlong microvilli that were more prominent at earlier than later stages. The microvillus brush border is marked by a white arrowhead in A, B, C and D. White dashed lines mark clone boundaries in all panels. Oc, oocyte; Fc, follicle cells. Numbers indicate stages of oogenesis.
Cad99C overexpression
Figure 25 In Cad99C-overexpressing follicle cells, the amount of Cad87A in the apical plasma membrane appears to be reduced.

The control fluorescence intensity ratios, the ratio of Cad87A fluorescence intensity in mutant cell / mutant cell (m/m) and the ratio of Cad87A fluorescence intensity in wild-type cell / wild-type cell (w/w) peak at 1.2. In contrast, the ratio of Cad87A fluorescence intensity in wild type cell / mutant cell, w/m, shows a broader distribution. Most w/m values are greater than 1.5, suggesting that the amount of Cad87A in the apical plasma membrane of Cad99C-overexpressing cells is reduced.
Anticad87A antibody fluorescence intensity in stage 10B follicle cells

Anticad87A antibody fluorescence intensity in stage 11 or older follicle cells
titrated away by the amount of Cad87A in mutant cells.

Next, Cad99C distribution in Cad87A-overexpressing cells was studied during stages 10B-11. At early or mid stage 10B, there appears to be an enrichment of Cad99C in microvilli as well as in the cytoplasm (Fig. 26A”, B”, C”, D”). A portion of the cytoplasmic Cad99C is in the form of vesicles. In addition, some of these Cad99C positive vesicles were also Cad87A-positive (Fig. 26A, B, C, D, yellow). However more experiments and quantification analysis are needed to confirm this observation.

Finally, Nomarki optics was used to analyze microvillus and vitelline membrane morphogenesis in follicle cells overexpressing Cad87A. During stage 10B, the regular stripe pattern of the brush border was lost as a result of Cad87A overexpression (Fig. 26E-E’, F-F’). In wild-type cells, the microvillus brush border of each follicle cell exhibits a dome-shaped profile shown by Nomarski optics (Fig. 26E). Fig. 26E’ shows that the microvillus brush border in Cad87-overexpressing cells does not have a dome-shaped profile. Vitelline bodies appear irregular in shape and distribution (Fig. 26F’). The mutant cell phenotype is reminiscent of the one observed when Cad74A was overexpressed (Fig. 15B). Nevertheless, a continuous vitelline membrane of apparently normal morphology is formed at stage 11 (Fig. 26G-G’). In conclusion, the defects in the layer of vitelline bodies suggest defects in the microvillus brush border, although Cad99C staining did not reveal obvious microvillus defects.

3.3 Overexpressing both Cad99C and Cad87A leads to long microvilli, similar to the long microvilli produced by overexpressing Cad99C alone

To analyze microvillus and vitelline membrane formation when Cad99C and Cad87A are both overexpressed, recombinant flies carrying both UAS-Cad99C-FL and UAS-Cad87A-FL were generated by meiotic recombination. Morphology of microvillus and vitelline membrane were analyzed using Nomarski optics. As shown in Fig. 27, at stage 10B, cells expressing both transgenic proteins produced longer microvilli than wild-type cells (Fig. 27A-A”). The phenotype is similar to overexpressing Cad99C alone. Microvilli in cells overexpressing both proteins displayed a fan-shaped profile and were equally spaced out (Fig. 27A-A”). The two transgenic proteins colocalized perfectly with each other in the microvilli, in cytoplasmic vesicles and along the lateral membranes of follicle cells (Fig. 27A, yellow). Anti-Cad99C
Figure 26 Overexpression of Cad87A appears to affect microvilli.

(A-D) show a confocal cross section of the follicular epithelium. (E-G) are Nomarski images. Transgenic protein Cad87A was expressed in follicle cell clones using Act5c>CD2>Gal4. Cad99C is in red. Cad87A is in green. (A, B, C, D) During stage 10B – stage 11, in Cad87A-overexpressing cells, there is a strong enrichment of Cad87A in the apical plasma membrane. (A’, B’, C’, D’) Cad87 is also ectopically localized to the lateral membranes of follicle cells, in the cytoplasm and in cytoplasmic vesicles. (A”, B”, C”, D”) During stage 10B – 11, in cells that overexpress Cad87A, there is an increased amount of Cad99C in microvilli as well as in the cytoplasm. Some of the Cad99C-positive cytoplasmic vesicles are also Cad87A-positive. (E-G) Nomarski images reveal that before the formation of the vitelline membrane, Cad87A overexpression disrupts the microvillus brush border. However, at stage 11, when the vitelline bodies have fused, no obvious defect is observed in vitelline membrane morphology. The microvillus brush border is marked by a white arrowhead in A, B, C, D, E, F, and G. White dashed lines mark clone boundaries in all panels. Oc, oocyte; Fc, follicle cells. Numbers indicate stages of oogenesis.
Cad87A overexpression
antibody did not stain the microvilli brush border in wild-type cells probably because they were titrated away by nearby cells expressing a great amount of Cad99C. Nomarski image (Fig. 27B) confirmed that in recombinant cells, microvilli were longer compared to those in wild-type cells and that the stripe pattern of microvilli was preserved in cells expressing both transgenic proteins. At stage 11, when both proteins were overexpressed, they were similarly colocalized in the microvilli, along the basolateral membranes, and in cytoplasmic vesicles (Fig. 27C–C”). In Nomarski image, microvilli separated by vitelline bodies were evident in cells expressing both transgenic proteins. In contrast, a smooth vitelline membrane was formed in wild-type cells by this stage (Fig. 27D). In conclusion, the phenotype obtained from expressing Cad99C and Cad87A is similar to when Cad99C was expressed alone.

3.4 Cad99C and Cad87A are part of the same protein complex

Kazmierczak et al. (2007) showed that the tip link is formed by Cdh 23 cis-homodimers interacting in-trans with Pcdh15 cis-homodimers at their N-termini. Because Cad99C and Cad87A are the homologues of the mammalian Pcdh15 and Cdh23, respectively, I wanted to investigate whether Cad99C and Cad87A could interact physically with each other. Toward this goal, a Co-IP experiment was carried out using ovaries, in which Cad99CΔcyt::GFP was expressed in follicle cells with the help of tj-Gal4. Cad99CΔcyt::GFP was immunoprecipitated by magnetically labelled anti-GFP antibodies, and the resulting eluates were analyzed by the anti-Cad87A antibody. As shown in Fig. 28A, Cad87A co-immunoprecipitated with Cad99CΔcyt::GFP (Fig. 28A, Lane 1). Although a weak signal was detected with the mCD8::GFP control (Fig. 28A, Lane 2), the signal intensity was much lower than that in Lane 1. Hence, the weak signal was likely a result of an incomplete wash of the column. The protein detected in Lane 1 was estimated to be ~219 kDa, in accordance with the predicted molecular mass of Cad87A (~217 kDa). Presence of Cad87A in all lysates before immunoprecipitation was confirmed by checking the original cell lysates with the anti-Cad87A antibody (Lanes 3 and 4, Fig. 28A). To determine whether the extracellular cadherin domains of Cad99C can interact with Cad87A independent of the Cad99C cytoplasmic tail or not, the same experiment was repeated using cell lysate that expressed Cad99CΔcyt::GFP in a Cad99C mutant background. Fig. 28B showed that the extracellular cadherin domains of Cad99C could interact with Cad87A in cells that were devoid of full length Cad99C, suggesting that the interaction was independent of the cytoplasmic tail of Cad99C (Lane 2, Fig. 28B). The band in Lane 2 corresponds to a protein size
Figure 27 Overexpressing both Cad99C and Cad87A leads to long microvilli, similar to the long microvilli produced by overexpressing Cad99C alone.

(A-A”, C-C”) show a confocal cross section of the follicular epithelium. (B, D) are the respective Nomarski images. (A-B) is a late stage 10B egg chamber. (C-D) is a stage 11 egg chamber. Transgenic proteins Cad99C and Cad87A were expressed in the follicle cell clones using Act5c>CD2>Gal4. Cad99C is in red. Cad87A is in green. (A-A”, C-C”) Overexpressing both Cad99C and Cad87A resulted in overlong microvilli that were equally spaced out, similar to the overlong microvilli produced when Cad99C was overexpressed alone. (B, D) Nomarski images showed that in mutant cells, vitelline bodies that were similar in size were separated by the overlong microvilli produced from overexpressing Cad99C and Cad87A. (A, C) Overexpressed Cad99C and Cad87A colocalized in the microvilli, along the basolateral plasma membranes of follicle cells and in cytoplasmic vesicles. The microvillus brush border is marked by a white arrowhead in A and C. White dashed lines mark clone boundaries in all panels. Oc, oocyte; Fc, follicle cells.
Cad99C and Cad87A overexpression
of ~ 215 kDa, consistent with the predicted molecular mass of Cad87A. No signal was detected in the negative control lane (Lane 1, Fig. 28B). Lanes 3-4 in Fig. 28B showed that Cad87A was present in cell lysates before immunoprecipitation, and that similar amounts of cell lysates were loaded.

To confirm that the GFP-tagged proteins were immunoprecipitated by the magnetically labeled anti-GFP antibodies, the immunoprecipitate was probed with an anti-GFP antibody. Fig. 28C shows that the GFP-tagged proteins were immunoprecipitated (Lanes 1, 2) and were present in the cell lysates before immunoprecipitation (Lanes 3, 4). Also, the GFP-tagged proteins in the eluate correspond in their apparent molecular masses (Cad99C∆cyt::GFP ~247 kDa, mCD8::GFP ~55 kDa) to the GFP-tagged proteins in the cell lysates (the bands in Lanes 1 and 2 correspond to the bands in Lanes 3 and 4, respectively). In summary, these results suggest that Cad87A and the extracellular cadherin domains of Cad99C can interact without the presence of the cytoplasmic tail of Cad99C. The fact that the extracellular cadherin domains of Cad99C did not pull down DE-Cad as shown in Fig. 22C supports that the Cad99C-Cad87A interaction is a specific one.
Figure 28 Cad87A interacts with the extracellular region of Cad99C, independent of the cytoplasmic tail of Cad99C.

(A) GFP-tagged proteins that were expressed in a wild-type background were immunoprecipitated from ovarian lysates by magnetically-labeled anti-GFP antibodies. Anti-Cad87A GP2 antibodies were used to detect Cad87A. Cad87A co-immunoprecipitated with Cad99C∆cyt::GFP (Lane 1), whereas only a weak signal was detected in the negative control lane (Lane 2). (B) Cad87A co-immunoprecipitated with Cad99C∆cyt::GFP that was expressed in a Cad99C<sup>21-5</sup> mutant background, indicating that the cytoplasmic tail of Cad99C was dispensable for the interaction. (C) Blot (B) was stripped and probed with anti-GFP antibodies to show that the immunoprecipitation was successful. Protein ladders are indicated by black solid lines at the left of each blot. Red asterisks indicate proteins of interest that are of the right sizes.
A anti-Cad87A

Co-IP

lysate

1, 3: mCD8::GFP
2, 4: Cad99CΔcyt::GFP & no endogenous Cad99C

B anti-Cad87A

Co-IP

lysate

1, 3: mCD8::GFP
2, 4: Cad99CΔcyt::GFP & no endogenous Cad99C

C anti-GFP

IP

lysate

1, 3: mCD8::GFP
2, 4: Cad99CΔcyt::GFP & no endogenous Cad99C
Chapter 4
Discussion

This work extends the functional analysis of Cad99C by identifying several interacting partners and studying their interactions in follicle cell microvilli. The interacting partners of Cad99C can be divided into two categories, intracellular and extracellular interacting partners. I will first discuss the Cad99C-MyoVIIA interaction, which is intracellular, followed by the interaction of Cad99C with the other two cadherins, Cad74A and Cad87A, which is extracellular. Finally, this chapter will end with the discussion of the roles of Cad99C, Cad74A, and Cad87A in vitelline membrane assembly.

1 MyoVIIA and Cad99C interact in follicle cell microvilli

1.1 Cad99C and MyoVIIA are part of the same protein complex

Previous work done in our lab strongly suggested that the cytoplasmic tail of Cad99C is necessary and sufficient to recruit MyoVIIA to the microvilli (Glowinski, MSc. Thesis 2008). This raised the question whether the two molecules interact via the cytoplasmic tail of Cad99C.

The biochemical experiments that I conducted provide strong evidence that the two proteins do indeed molecularly interact. Moreover, the interaction is mediated by the cytoplasmic tail of Cad99C because MyoVIIA co-immunoprecipitated with Cad99C∆ex::GFP. The interaction is further supported by the reverse experiment, which showed that MyoVIIA::GFP can pull down Cad99C.

Though the experiments conducted so far provide compelling evidence that Cad99C and MyoVIIA interact, there was one puzzling observation. As mentioned previously, Cad99C∆ex::GFP, when overexpressed in follicle cells, caused short microvilli. Moreover, a great number of vesicles positive for Cad99C∆ex::GFP and endogenous Cad99C were seen in the cytoplasm. This indicates that Cad99C∆ex::GFP acts in a dominant-negative manner in causing a turnover of endogenous Cad99C (Liu and Godt, unpublished observations). Also in Cad99C∆ex::GFP-expressing cells, there were vesicles that are positive for MyoVIIA. Surprisingly, these vesicles did not contain Cad99C∆ex::GFP (Glowinski and Godt, unpublished observations). As MyoVIIA and Cad99C interact in follicle cell microvilli, we might expect
them to be recycled together. However this observation seems to suggest otherwise. It could be that the two molecules interact in the microvilli, however, when they are being degraded or recycled, they are separated into different vesicles because the pathways involved in the degradation or recycling are different. More experiments are required to test this.

In vertebrates, a physical interaction between murine MyoVIIA and Pcdh15, the orthologues of *Drosophila* MyoVIIA and Cad99C, respectively, was demonstrated by Senften *et al.* (2006) biochemically. Using GST pull down assays, Senften *et al.* (2006) showed that MyoVIIA and Pcdh15 participate in a direct physical interaction that was mediated by the SH3 domain of MyoVIIA and the cytoplasmic domain of Pcdh15. The minimal consensus sequence of a SH3 domain binding site is PxxP (reviewed by Kaneko *et al.*, 2008), and the cytoplasmic domain of Pcdh15 contains 4 such motifs (Senften *et al.*, 2006). The cytoplasmic tail of Cad99C has diverged substantially from its mammalian orthologue (D’Alterio *et al.*, 2005), and is rather proline poor. There is one PxxP motif (PTGP) at amino acid positions 1596-1599 in the Cad99C cytoplasmic tail. However, this PxxP motif is not conserved among insects (*Anopheles gambiae, Apis mellifera, Tribolium castaneum*). Hence, it seems unlikely that PTGP is a real SH3 domain binding site. If this is true, then the MyoVIIA-Cad99C interaction is most likely indirect, unlike the interaction between their mammalian orthologues. To see whether a direct interaction exists or not, purified Cad99C and MyoVIIA can be used in pull down assays. To see whether the PTGP motif is responsible for a direct physical interaction, the PTGP sequence can be mutated and the mutated and normal proteins can be subjected to pull down assays.

In conclusion, previous functional analysis of MyoVIIA together with my work strongly suggest that MyoVIIA and Cad99C function in a common process that regulates microvillus morphogenesis.

### 1.2 MyoVIIA regulates microvillus density

Previous work in our lab has demonstrated that MyoVIIA is required for a normal microvillus structure (Glowinski, MSc. Thesis 2008). In follicle cells that are homozygous for a MyoVIIA null allele, microvilli are shorter, thinner and show defects in differential growth such that all microvilli are equal in size when compared to wild-type microvilli. Because the microvillus core is essentially F-actin, and MyoVIIA associates with F-actin structures during the course of
As an extension to our ongoing studies of MyoVIIA function in microvillus morphogenesis, I performed a gain-of-function analysis on MyoVIIA and analyzed the effects on Cad99C and follicle cell microvilli. When MyoVIIA was overexpressed, Cad99C was abnormally concentrated in the basal domain of microvilli, where MyoVIIA is highly enriched, and was reduced in the remaining microvillus. Conversely, overexpression of Cad99C recruits excessive MyoVIIA protein to the microvillus (Glowinski, MSc. Thesis 2008). Thus, higher than normal concentration of either molecule leads to redistribution of the other molecule, suggesting that these two molecules can recruit each other and that a particular ratio of Cad99C to MyoVIIA is needed for normal subcellular distribution and function.

Furthermore, overexpressing MyoVIIA causes microvilli to be thicker, and more spaced out from each other than in wild type (Godt, unpublished observations). This is consistent with the MyoVIIA mutant analysis mentioned above, where microvilli were thinner and more densely packed (Glowinski, MSc. Thesis 2008). These observations point to a potential role of MyoVIIA in organizing the spatial distribution of microvilli. In other words, MyoVIIA may regulate the density of follicle cell microvilli.

In support of a role of MyoVIIA in regulating microvillus density, in ck mutant escapers, hairs (F-actin protrusions) on the body and wing are split into numerous ones (Kiehart et al., 2004). Moreover, the wing hairs and body hairs of mutant escapers are thinner and shorter, respectively, than wild-type hairs. These observations suggest that MyoVIIA acts in positioning the actin bundles that will give rise to mature hairs (Kiehart et al., 2004).

In conclusion, we propose that MyoVIIA regulates the density of microvilli in follicle cells of the Drosophila ovary. MyoVIIA may provide nucleation points for microvillus formation by recruiting Cad99C as well as F-actin.

To further test this hypothesis, it would be important in the future to determine the microvillus density in follicle cells that express either full-length Cad99C or the truncated isoform of Cad99C that lacks the cytoplasmic region. If our hypothesis is correct, we would expect the density of microvilli in follicle cells that express full-length Cad99C be different from that of
cells expressing extracellular domains of Cad99C, since in the latter case, the cytoplasmic region is missing, thus there will be no interaction with MyoVIIA. Consequently, MyoVIIA cannot regulate the density of microvilli through Cad99C.

2 Cad99C-Cad74A and Cad99C-Cad87A interactions in follicle cell microvilli

2.1 Summary

To look for other microvillus regulators in addition to Cad99C, I performed gain-of-function and loss-of-function analyses on Cad74A and Cad87A, two other cadherins that are also located in follicle cells microvilli. A comprehensive summary is listed in Table 1. The data collected still provide no clear evidence for any functional roles of these two cadherins in follicle cell microvilli. However, the following conclusions can be drawn:

1) Cad74A is not essential for maintaining the microvillus structure or forming a normal vitelline membrane. When Cad74A was reduced to undetectable levels in follicle cells, only a slight change in Cad99C concentration or no defects in vitelline membrane morphology was observed. This is consistent with results reported by Zartman et al. (2008). The authors tested whether the vitelline membrane of eggs that are homozygous for a Cad74A null allele still functions normally in restricting permeability by first removing the outer chorion, then staining the eggs with Neutral Red. They found that the mutant eggs were impermeable to the dye, suggesting that the vitelline membrane functions normally (Zartman et al., 2008). Moreover, my data suggest that downregulating Cad74A in cells that are Cad99C-deficient did not worsen the microvillus/vitelline membrane phenotype compared to cells that are Cad99C negative but Cad74A positive.

2) The gain-of-function effect of Cad74A on microvilli depends on the presence of Cad99C. Despite the long, disorganized microvilli that resulted when Cad74A::GFP was overexpressed in a wild-type background, in follicle cells that were devoid of Cad99C, overexpressing Cad74A was not able to produce apical protrusions. In contrast, the potential of Cad99C in promoting microvillus growth was not compromised by the absence of Cad74A.
<table>
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<tr>
<th>Genotype of tissue used</th>
<th>Change in protein expression level</th>
<th>Result</th>
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<tr>
<td><strong>Cad99C-MyoVIIA interaction</strong></td>
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<tr>
<td>HsFLP/UAS-ck::GFP; Act5c&lt;CD2&lt;Gal4/</td>
<td>MyoVIIA overexpression</td>
<td>Cad99C enriched in basal region of microvilli.</td>
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<td><strong>Cad99C-Cad74A interaction</strong></td>
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<td>hsFLP; Act5c&lt;CD2&lt;Gal4/UAS-Cad99C</td>
<td>Cad99C overexpression</td>
<td>• Overlong microvilli</td>
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<td>• Cad74A localized to the overlong microvilli</td>
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<td>• Apical Cad74A reduced</td>
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<td>hsFLP; Act5c&lt;CD2&lt;Gal4/UAS-Cad74::GFP</td>
<td>Cad74A overexpression</td>
<td>• Overlong and disorganized microvilli</td>
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<td></td>
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<td>• Cad99C reduced in the microvilli</td>
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<tr>
<td>hsFLP; Act5c&lt;CD2&lt;Gal4/ UAS-Cad99C UAS Cad74::GFP</td>
<td>Cad99C overexpression Cad74A overexpression</td>
<td>• Abnormally long and disorganized microvilli</td>
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<td>• Cad99C and Cad74A colocalized in the microvilli</td>
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<td>hsFLP; Act5c&lt;CD2&lt;Gal4/ UAS-Cad99C^RNAi</td>
<td>Cad99C reduction</td>
<td>• Defective microvilli and vitelline membrane</td>
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<tr>
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<td>• Cad74A is reduced at late stage 10B and shows a different distribution at stage 11 and onwards</td>
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<tr>
<td>hsFLP; Act5c&lt;CD2&lt;Gal4/ UAS-Cad74A^RNAi</td>
<td>Cad74A reduction</td>
<td>No effect on vitelline membrane morphology</td>
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<td>The vitelline membrane defects are similar to the ones caused by Cad99C^RNAi alone.</td>
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<td>hsFLP; Act5c&lt;CD2&lt;Gal4/ UAS-Cad99C^RNAi UAS-Cad74A^RNAi</td>
<td>Cad99C overexpression Cad74A reduction</td>
<td>Overlong microvilli similar to Cad99C overexpression alone.</td>
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<td>No microvilli even though Cad74A is overexpressed.</td>
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<td><strong>Cad99C-Cad87A interaction</strong></td>
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<td>hsFLP; Act5c&lt;CD2&lt;Gal4/ UAS-Cad99C</td>
<td>Cad99C overexpression</td>
<td>• Overlong microvilli</td>
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<td>• Cad87A localized to the overlong microvilli</td>
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<td>• Apical Cad87A reduced</td>
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<td>hsFLP; Act5c&lt;CD2&lt;Gal4/ UAS-Cad87A</td>
<td>Cad87A overexpression</td>
<td>• There may be defects in the interface zone between the oocyte and follicle cells.</td>
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<td>• More Cad99C in microvilli and in the cytoplasm</td>
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<td>hsFLP; Act5c&lt;CD2&lt;Gal4/ UAS-Cad99C UAS-Cad87A::GFP</td>
<td>Cad99C overexpression Cad87A overexpression</td>
<td>• Overlong microvilli similar to Cad99C overexpression alone</td>
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<td>• Cad99C and Cad87A colocalized in the microvilli</td>
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Table 1. Summary of immunostaining experiments.
3) Cad74A and Cad99C appear to negatively regulate each other in follicle cells. First, for late stage 10B follicles, there appears to be a reduction of Cad74A on the apical surface of cells that overexpressed Cad99C. However, the reduction in Cad74A was not visible in later stages when Cad74A levels are high in wild-type follicle cells. An antagonistic effect of Cad99C may not be detectable at later stages due to high levels of Cad74A. Second, overexpressing Cad74A caused overlong, disorganized microvilli. The ability of Cad74A in producing disorganized microvilli was not abolished even at high concentrations of Cad99C. The phenotype that resulted from overexpressing both proteins appears to be additive, suggesting separate functions for these cadherins in microvilli.

4) Overexpressing Cad99C at a stage when Cad87A is expressed at low levels appeared to reduce the amount of Cad87A at the apical surface, similar to Cad74A. In the reverse experiment, overexpressing Cad87A also seemed to have a negative effect on Cad99C and microvilli. Nomarski images revealed that in Cad87A-overexpressing cells, the microvillus morphology was abnormal. However, unlike Cad74A, the negative effect of Cad87A overexpression on follicle cell microvilli can be overcome by Cad99C overexpression.

2.2 The extracellular domains of Cad99C interacts with Cad74A and Cad87A

Biochemical experiments demonstrated that Cad99C and Cad74A, and Cad99C and Cad87A can interact and that the interactions are independent of the Cad99C cytoplasmic tail. The Cad99C-Cad74A interaction was demonstrated by the following experiments: 1) Cad99C∆cyt::GFP, a truncated isoform of Cad99C in which the cytoplasmic tail is removed, could pull down Cad74A when expressed in a wild-type or a Cad99C mutant background. 2) Cad74A::GFP pulled down Cad99C, and 3) Cad99C∆cyt::GFP did not pull down DE-Cad, showing that the cadherin domains of Cad99C do not generally bind to any other cadherin, which supports the specificity of the Cad99C-Cad74A interaction. Similarly, the Cad99C-Cad87A interaction was shown by the fact that Cad99C∆cyt::GFP was able to pull down Cad87A in both wild-type and Cad99C mutant backgrounds, but not DE-Cad.

Furthermore, several findings suggest that both Cad99C and Cad74A might be glycosylated, which is a common feature of membrane proteins in eukaryotic cells (reviewed by Peter-Katalinic, 2005). First, anti-Cad99C and anti-Cad74A antibodies consistently recognized
proteins that are considerably bigger than the predicted sizes for Cad99C and Cad74A, respectively. Secondly, there is data supporting that there is N-linked glycosylation of Cad99C in the extracellular region (D’Alterio et al., 2005). Third, potato lectin, might bind directly to Cad99C and Cad74A, as shown by the precise colocalization between potato lectin and these cadherins in wild-type follicle cells and follicle cells that overexpress these cadherins (data not shown). Interestingly, Cad74A primarily interacts with high-molecular isoforms of Cad99C that constitute a minor fraction of total Cad99C, as these isoforms were only detectable in co-immunoprecipitates in which Cad74A was overexpressed. Hence, I hypothesize that these isoforms might represent particular glycosylated isoforms of Cad99C.

The mammalian homologues of Cad99C and Cad87A/Cad74A, Pcdh15 and Cdh23, respectively were also shown to interact (Kazmierczak et al., 2007). In mature cochlear stereocilia, Cdh23 and Pcdh15 homodimers interact via their N-termini to form the upper and lower end of the tip link, respectively. During development of the hair bundle in the mouse inner ear, Cdh23 and Pcdh15 have been localized to the transient lateral links and kinociliary links (Ahmed et al., 2006; Senften et al., 2006; Siemens et al., 2004). Moreover, hair bundles of mouse mutants carrying mutated alleles of these two genes are fragmented (waltzer and Ames waltzer for Cdh23 and Pcdh15, respectively), suggesting that Cdh23 and Pcdh15-mediated adhesion contributes to the formation of the cohesive hair bundles (Lefevre et al., 2008). In addition, Cdh23 and Drosophila Cad74A had been shown to mediate homophilic adhesions in vitro (Kazmierczak et al., 2007; Lovegrove et al., 2006; Siemens et al., 2004).

From the Cad74A overexpression experiments I conducted, there is no indication of homophilic cell-cell adhesion between plasma membrane of adjacent microvilli that is mediated by Cad74A. Cad74A is not enriched in the lateral membrane between adjacent Cad74A::GFP expressing cells compared to the lateral membrane between wild-type and those that express Cad74A::GFP. If Cad74A were to mediate homophilic adhesion, then an enrichment of Cad74A at the lateral membrane between Cad74A::GFP expressing cells would be expected. The same reasoning and conclusion can be made for Cad87A. Similarly, it has been proposed that Cad99C does not participate in homophilic adhesive interactions between microvilli (D’Alterio et al., 2005).

Nevertheless, whether Cad99C, Cad74A, and Cad87A form cis- and/or trans-heterodimers remains an open question. As mentioned above, Pcdh15 and Cdh23 form trans-heterodimers in
forming the tip link (Kazmierczak et al., 2007). It is possible that the *Drosophila* homologues may participate in such interactions. Further experiments are needed to test this possibility.

Recently, the hair bundles of the *waltzer* and *Ames waltzer* mice have also been shown to have defects in the differential elongation of stereocilia during early developmental stages, suggesting that Cdh23 and Pcdh15 also have a role in regulating F-actin assembly in the stereocilium core (Lefevre et al., 2008). Whether Cad87A and Cad74 have an evolutionarily conserved role in regulating actin assembly is still unclear. It will be important in the future to determine whether and how the cadherin interactions shown by biochemical experiments can be translated into functions in follicle cell microvillus morphogenesis or eggshell formation.

### 2.3 Limitations of the functional analysis of Cad74A and Cad87A

Given that Cad74A and Cad87A localize to microvilli, and that both biochemically interact with Cad99C, we would expect to see an interaction of each cadherin with Cad99C at the cellular level. However, the analysis discussed above shows no clear evidence for it. One possibility is that these molecules do not interact at the functional level. Another possibility is that methods used for analysis were not sensitive enough to detect the effects. Below, I list some limitations of my analysis:

1) The analysis of microvillus morphology in many of my experiments was confounded by using Cad99C as a marker for microvilli. A marker for microvilli that is not influenced by the cadherins is needed to objectively analyze microvillus morphology.

Throughout my analysis, microvillus morphology was also evaluated with the help of Nomarski images, a Cad99C independent approach. By carefully examining Nomarski images and comparing with Cad99C stainings, it was revealed that microvilli are displayed as depressions on Nomarski images and that vitelline bodies are situated in between (D’Alterio et al., 2005; Schlichting et al., 2006). For example, in Fig. 15, the microvilli revealed by Cad99C staining (Fig. 15 A’) matched the depressions on the respective Nomarski image (Fig. 15 A, B). However, Nomarski optics often gives images that are difficult to interpret.

In an attempt to circumvent this problem, lectins from *Solanum tuberosum* (potato) were used to mark microvilli. Lectins are glycoproteins that bind to carbohydrate groups of polysaccharides,
glycoproteins, and glycolipids (reviewed by De Mejia and Prisecaru, 2005; Rudiger and Gabius, 2001). Unfortunately, lectins from Solanum tuberosum seem to label Cad74A (data not shown). In follicle cells that expressed Cad74A::GFP, potato lectin colocalized precisely with the Cad74A::GFP protein, suggesting that this lectin binds to Cad74A, and cannot be used as an independent marker for microvilli.

Another alternative for analyzing microvillus/vitelline membrane morphology would be to prepare histological sections of egg chambers. Histological sections can provide useful information that can not be obtained from whole mounts. For example, in Cad99C null egg chambers, histological sections revealed holes in the vitelline membrane at stage 11 (D’Alterio et al., 2005). In contrast, whole mounts, holes were not observed. In addition, the analysis of follicle cell microvilli would benefit from the use of electron microscopy, which has a higher resolution than light microscopy, since microvilli are very fine structures.

2) One caveat with the gain-of-function analysis is that when the protein level varies significantly from its normal concentration, which might result in abnormal molecular interactions. Moreover, we should be cautious with overexpression analysis, in which a protein is ectopically expressed spatially and temporally, that is, overexpressing a protein at a time and place when it is not normally present. For this work, Cad99C was ectopically expressed at high levels at late stages of oogenesis when its expression level is normally low (D’Alterio et al., 2005). Also, for Cad74A gain-of-function analysis, Cad74A::GFP was prematurely expressed at stage 10A, a stage when it is not normally expressed in wild type.

3) The GFP tag of Cad74A::GFP is at the C-terminus, which contains a putative PDZ-domain binding site. Therefore, the GFP tag may potentially block interaction partners, and consequently alter the function of the wild-type protein. One should be cautious when interpreting results from the use of the UAS-Cad74A::GFP construct.

4) The Co-IP experiments conducted for the cadherins were done in the presence EDTA, which chelates Ca\(^{2+}\) ions. For cadherins to form a rigid structure and participate in homophilic and heterophilic binding, Ca\(^{2+}\) is needed. Therefore, in principle we would not expect to see an interaction between the cadherins. However, as shown in the Results section, Cad99C can pull down Cad74A and Cad87A. One possible explanation for this could be that EDTA did not chelate all Ca\(^{2+}\) in follicle cells, and some residual Ca\(^{2+}\) mediated the interaction between
Cad99C-Cad74A and Cad99C-Cad87A. Nevertheless, to confirm that the extracellular region of Cad99C does not pull down DE-Cad, the Co-IP experiment should be repeated in the absence of EDTA.

3 The roles of Cad99C, Cad74A, and Cad87A on microvillus and vitelline membrane morphogenesis

Gain-of-function and loss-of-function analysis on Cad99C and Cad74A shows that firstly, Cad99C is essential for normal microvillus and vitelline membrane formation, and its function cannot be substituted by Cad74A. This is supported by the following observations: 1) Downregulation of Cad99C by RNAi produced defects in the vitelline membrane, consistent with published findings (D’Alterio et al., 2005). 2) Overexpressing Cad74A in the absence of Cad99C cannot rescue defects in microvillus and vitelline membrane morphology.

Secondly, the function of Cad99C in promoting microvillus growth does not depend on Cad74A. Cad99C-overexpressing follicle cells form overlong microvilli, which persist to late stages of oogenesis (D’Alterio et al., 2005; this work). This potential is maintained in follicle cells that are Cad74A deficient. Finally, Cad99C localization in microvilli does not depend on Cad74A as in Cad74A deficient cells, the localization of Cad99C was not affected and the amount of Cad99C only slightly reduced.

Thirdly, Cad99C may be the major molecule functioning in the formation of microvilli and in early stages of vitelline membrane assembly during stage 9-10. However as oogenesis proceeds, Cad74A and Cad87A may become involved in the formation of subsequent eggshell layers. In wild-type egg chambers, Cad99C expression is highest during stage 10A of oogenesis. As oogenesis proceeds, microvilli regress and Cad99C expression decreases. The reduction of Cad99C during later stages may be needed for the high levels of Cad74A and Cad87A to accumulate in follicle cell microvilli, because in Cad99C-overexpressing cells, the amount of Cad74A and Cad87A in the apical plasma membrane is reduced.

3.1 The role of Cad99C in vitelline membrane assembly

Studies done by Elalayli et al. (2008) on a minor vitelline membrane protein, Palisade, offer us insight into the role of Cad99C in vitelline membrane assembly, as both palisade and Cad99C
null mutants show phenotypic similarities. Firstly, both Cad99C and Palisade potentially interact with vitelline membrane proteins to help keep them in place. In Cad99C and palisade mutants, one of the major vitelline membrane proteins, sV17, was abnormally taken up by the oocyte (Elalayli et al., 2008). However, not all vitelline membrane proteins were affected in the same way as sV17, because the localization of another major vitelline membrane protein, sV23, was normal in Cad99C mutants. Nevertheless, there are potentially other vitelline membrane proteins that could well be similarly affected. Secondly, microvilli and vitelline bodies in these mutants showed disorganization at stage 10 (Elalayli et al., 2008). And lastly, both mutants are defective in the subsequent disulfide cross-linking of vitelline membrane proteins.

Nonetheless, Cad99C and palisade null mutants differ in the severity of the defects. For one thing, the microvillus defect seen in Cad99C mutants is far greater than in palisade mutants, as microvilli are not only disorganized, they are barely detectable. This is consistent with the fact that Cad99C is an essential component of follicle cell microvilli (D’Alterio et al., 2005; Schlichting et al., 2006). On the other hand, palisade mutants showed more severe defects in the processing and crosslinking of the vitelline membrane proteins, which occur later during oogenesis (after the formation of the microvilli) (Elalayli et al., 2008). Furthermore, loss of Cad99C has a more deleterious effect on the eggshell than loss of Palisade. Only <2% of the eggs laid by Cad99C mutant females produced larvae whereas 70-80% of the eggs from palisade mutant females survived to the larval stage. This can be explained by the observation that the vitelline membrane in Cad99C mutant egg chambers does not recover during late oogenesis leading to the desiccation of eggs (D’Alterio et al., 2005; this work), whereas the gaps in palisade mutants are repaired by stage 13 (Elalayli et al., 2008).

Taken together, Elalayli et al. (2008) proposed that Cad99C and Palisade act in a common pathway for the formation of a proper eggshell. However Cad99C acts earlier in the pathway in that its primary role is to form a scaffold for vitelline membrane material, while Palisade coordinates processes that occur later by interacting with proteins on the scaffold and possibly with the scaffold. Consistent with this hypothesis, in Cad99C mutants, there appears to be a reduction in Palisade levels, though the reduction was variable, whereas no difference in Cad99C levels was detected in palisade mutants compared to controls (Elalayi et al., 2008). In addition, no defect in vitelline membrane morphology was observed in egg chambers that were heterozygous for Cad99C and palisade null alleles. The system might be more robust than
expected and other vitelline proteins might act accordingly to compensate for the loss of half of Cad99C and Palisade. Hence, it is worthwhile to study the extent to which the two proteins interact at the structural and functional level. Lastly, another question worth investigating is whether Cad99C and Palisade physically interact with sV17 and/or other vitelline membrane proteins to further decipher the mechanism through which sV17 is secured in the perivitelline space.

3.2 Cad74A and Cad87A function in follicle cells

As mentioned previously, at late stages of oogenesis, Cad74A is present in high amounts on the apical surface of follicle cells. Interestingly, it is localized in a broader region and more apical than Cad99C. Moreover, it is distributed as a thick layer on the apical surface, different from the spiky Cad99C distribution pattern, characteristic of microvilli. This raises the possibility that during late stages, Cad74A may get cleaved off from the plasma membrane, and contributes to the vitelline membrane material. In intestinal microvilli, it has recently been shown that the microvillar tips shed off vesicles of proteins into the intestinal lumen (McConnell et al., 2009). It would be interesting to determine whether a similar process occurs in the follicle cell microvilli during vitelline membrane formation.

As discussed in the Introduction, in the Drosophila ovary, Cad74A was shown to have a role in dorsal appendage formation (Zartman et al., 2008). Both loss-of-function and gain-of-function of Cad74A specifically in the roof cells produced deformed dorsal appendages. Forming a tube-like structure requires the roof cells to bend out of the plane of the follicular epithelium, which depends on the constriction of the apical domain of these cells. Because these processes are disrupted in both Cad74A loss-of-function and gain-of-function mutants, Zartman et al. (2008) suggest that Cad74A could have a role in modulating surface tension along the apical domain or in stabilizing cell-cell connections in the roof cells. Cad74A might have a similar function in other main body follicle cells, as Cad74A is also localized to the apical surface of these cells and to apical microvilli, which are mostly plasma membrane wrapped around F-actin cores. Because Cad74A and Cad87A are expressed at a stage when microvilli are regressing, they may help maintain tension in the plasma membrane of the microvilli during the regression, so that they do not collapse against the apical surface.
Another potential function of Cad74A and Cad87A would be a role in anchoring chorion proteins, as they are present at high levels on the apical surface of follicle cells during a stage when the chorion is made (stages 11-14) (Waring, 2000; Zartman et al., 2008).

If Cad74A and Cad87A indeed serve functions proposed above, then it is quite surprising that no obvious defects were observed when the protein levels were reduced (this work; Hwang and Godt, unpublished). One possibility is that the system Cad74A and Cad87A participate in is robust enough to buffer the perturbations. Cad74A and Cad87A might be redundant in function to each other and to the microvillus cadherin Cad88C, which is also a homologue of mammalian Cdh23 (Hwang and Godt, unpublished). Hence it is important to analyze the effects on follicle cell microvilli when all of the three genes are downregulated. Another possibility is that the techniques and equipment we are employing to study these molecules are not sensitive enough to detect the effects. Microvilli are very fine structures, and Cad74A and Cad87A show dynamic expression profiles and appear to have complex functions.

4 Working model

I extended the functional analysis of Cad99C by looking for intracellular and extracellular interacting partners of Cad99C. Inside the cell, MyoVIIA binds either directly or indirectly to the cytoplasmic domain of Cad99C (Fig. 29A). This interaction is probably particularly important during early stages of microvillus formation because MyoVIIA, which might influence the sites of F-actin nucleation, couples Cad99C to F-actin.

Extracellularly, the cadherin repeats of Cad99C interact with Cad74A and Cad87A, either forming cis- or trans-heterodimers (Fig. 29B, C). These interactions may further stabilize the plasma membrane by maintaining tension. Moreover, the microvillus cadherins may interact with other extracellular matrix proteins to coordinate the elongation and shortening of microvilli and formation of eggshell layers (Fig. 29 D).

In conclusion, I identified intracellular and extracellular components of the Cad99C protein complex, a critical regulator of microvillus morphogenesis, and contributed to a better understanding of the interactions between microvillus proteins.
Figure 29 Model of Cad99C function in follicle cell microvilli.

(A) MyoVIIA interacts directly or indirectly with the cytoplasmic domain of Cad99C. (B, C) The extracellular region of Cad99C interacts with Cad74A and Cad87A, independent of its cytoplasmic domain. These cadherins could form cis- (B) and/or trans- (C) heterodimers. (D) Cad99C potentially interacts with vitelline membrane components, such as Palisade or sV17.
The diagram illustrates the interaction between the oocyte and follicle cells. The following components are highlighted:

- **MyoVIIA**
- **Cad99C**
- **Cad74A/87A**
- **F-actin**

The diagram also indicates potential adaptor proteins linking MyoVIIA and Cad99C, as well as vitelline membrane components.
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