Examining the Function of β-catenin at Adherens Junctions during Dorsal Closure and the Formation of the Ventral Epidermis in *Drosophila*

by

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2015

Abstract

Adherens junctions (AJs) consist of Cadherin-Catenin Complexes (CCCs); each CCC contains DE-cadherin, p120catenin, β-catenin (Armadillo in *Drosophila*) and α-Catenin. The question addressed here was whether β-catenin functions primarily to physically link DE-cadherin to α-Catenin or it has additional regulatory function in AJ stability.

Dorsal closure (DC) defects in cadherin-deficient embryos are partially rescued by fusion proteins that directly link DE-cadherin to α-Catenin without recruiting Armadillo/β-catenin to AJs. This suggests that Armadillo at AJs is important for DC apart from being a linker. However, ventral defects were completely rescued by the same chimeras, indicating that Armadillo/β-catenin has no essential regulatory function at AJs in the ventral epidermis. Lastly, DC defects in Armadillo/β-catenin-deficient embryos were only partially rescued by the expression of DE-cadherin α-Catenin chimeras perhaps due to compromised Wnt signalling. Therefore, Armadillo/β-catenin has a general essential function in linking DE-cadherin to α-Catenin but a tissue-specific role in controlling AJ dynamics.
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<td>aa</td>
<td>amino acid(s)</td>
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<td>AEL</td>
<td>after egg laying</td>
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<td>AJ</td>
<td>Adherens junction</td>
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<td>Arm</td>
<td>Armadillo; the β-catenin homologue in <em>Drosophila</em></td>
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<td>Baz</td>
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<td>Cadherin-catenin complex</td>
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<td>Crb</td>
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<td>DEcad</td>
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<tr>
<td>PBST</td>
<td>phosphate buffer saline with 0.3% tris (hydroxymethyl) aminoethane</td>
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<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<td>wt</td>
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Chapter 1

Introduction

The purpose of this study was to examine the function of β-catenin at adherens junctions during dorsal closure and the formation of the ventral epidermis. Although previous studies have looked into the role of β-catenin at adherens junctions, it remains controversial whether β-catenin acts simply as a linker between DE-cadherin and α-Catenin or has other regulatory roles essential for adherens junction function. To answer this question, I rescued defects in dorsal closure and ventral epidermis in shotgun and armadillo mutant embryos using fusion proteins that directly link DE-cadherin to α-Catenin and lack the Armadillo binding site.

1.1 Epithelium

Epithelial tissues cover the surface of the body, and form major components of organs and blood vessels. They are formed by a continuous sheet of polarized cells that are tightly adhered to each other. The plasma membrane of epithelial cells is characterized by the presence of distinct apical and basolateral domains. These domains are differentiated by a distinct distribution of proteins and lipids. The apical domain faces the lumen and is important for the secretion and absorption of molecules while the basolateral domain adheres to neighboring cells and interacts with the basement membrane. The interface between the apical and basolateral domains is marked by cell-cell junctions. There are two types of cell-cell junctions at the apico-lateral membrane. The first type is the septate junction (in invertebrates) or tight junction (in vertebrates) which prevent the diffusion of molecules through the paracellular space. The second type is the adherens junction. Adherens junctions coalesce to form a belt of adhesion called the zonula adherens and physically link neighbouring cells, giving epithelia structural integrity (Figure 1) (Tepass 2012;

1.2 Adherens Junctions

Adherens junctions were first identified through electron microscopy as dense plaques at cell-cell contacts located on the cytoplasmic side of opposing cells (Farquhar and Palade, 1963). These cytoplasmic plaques are primarily formed by actin filaments (Hirokawa and Heuser, 1981; Miyaguchi 2000). Adherens junctions are important to maintain epithelial integrity by connecting the actin cytoskeletons of adjacent cells and regulating the organization of the membrane-associated actin and microtubule cytoskeleton. They are stable enough to withstand forces associated with tissue morphogenesis and are also dynamic, such that they assemble and reassemble when cells undergo cell-cell rearrangements, cell shape changes or division (Harris and Tepass, 2010, Harris 2012, Meng and Takeichi 2009; Gumbiner 2005). Two types of complexes are found at adherens junctions: the Nectin-Afadin complex and the Cadherin-Catenin Complex (Figure 1).

1.2.1 Nectin-Afadin Complex

Nectins are transmembrane immunoglobulin domain proteins found at adherens junctions (Takahashi et al. 1999). They can mediate both homophilic and heterophilic adhesion (Sakisaka et al. 2007). In some cell types, nectins are important for recruiting E-cadherin to adherens junctions and the formation of adherens junctions (Honda et al., 2003). The C-terminus of Nectin binds to Afadin via its PDZ (PSD-95/Dlg/zona occludens-1) homology domain (Mandai et al. 1997). Afadin then in turn connects Nectin to the actin cytoskeleton either directly or indirectly
through other actin-binding proteins such as Zonula Occludens-1 or α–catenin (Takai et al. 2008; Miyoshi and Takai 2005). There is one Drosophila homologue of Afadin, Canoe (Miyamoto et al. 1995), and three Nectin homologues: Echinoid, Roughest and Hibris. Canoe binds Echinoid and links it to filamentous actin. Echinoid is a component of most adherens junctions in fly epithelia. However, Echinoid is not required for adherens junction formation or maintenance (Sawyer et al. 2009; Sawyer et al. 2011). Differential expression of Echinoid is known to be important for cell sorting in the wing imaginal disc and in dorsal closure (Wei et al. 2005; Lin et al. 2007; Laplante and Nilson 2006). Additionally, Canoe is not essential for adherens junction assembly. However, Canoe is required to control morphogenetic movements like mesoderm invagination and germ band extension, in which it is necessary for regulating the link between adherens junctions and actin during apical constriction and for cell shape changes that are necessary for these processes to occur (Sawyer et al. 2009; Sawyer et al. 2011).

1.2.2 Cadherin-Catenin Complex

The Cadherin-Catenin Complexes consists of E-cadherin, p120catenin, β-catenin and α-Catenin. Cadherins were first identified in vertebrates for their ability to mediate calcium-dependent adhesion in cultured cells and for their role in forming epithelia in the early mouse embryo (Takeichi 1991). All cadherins have extracellular cadherin repeats that undergo calcium-dependent homophilic adhesion. Each extracellular cadherin domain consists of 110 residues and are structurally similar to immunoglobulin domains (Harris and Tepass 2010; Oda and Takeichi 2011; Meng and Takeichi 2009). The first members of the cadherin superfamily to be identified were the classic cadherins such as E-cadherin. They have a highly conserved cytoplasmic tail that interacts with two main catenins; p120 catenin and β-catenin. Non-classic cadherins include Flamingo, Dachsous, Fat, protocadherin 15, or Cadherin 23. Both classic and non-classic
cadherins have two or more extracellular cadherin domains, but non-classic cadherins lack the cytoplasmic tail that can interact with catenins. Genomic sequencing reveals genes encoding classic cadherins in primitive animals such as cnidarians and sponges (Adamska et al. 2007; Abedin and King 2008; King et al. 2008; Fahey and Degnan 2010; Chapman et al. 2010; Harris and Tepass 2010). Among the vertebrate classic cadherins, E-cadherin is found in almost all epithelial tissues, N-cadherin is found majorly in neural tissues and VE-cadherin is found in endothelial tissues (Oda et al. 2005; Hulpiau and van Roy 2009). Drosophila expresses three classic cadherins, DE-cadherin, DN-cadherin and Dncadherin2.

E-cadherin or DE-cadherin are transmembrane proteins that bind homophilically to cadherins on adjacent cells in a calcium-dependent manner (Takeichi 1991; Oda et al. 1994). DE-cadherin is encoded by shotgun (shg) in Drosophila (Tepass et al. 1996; Uemura et al. 1996). Mutants for shg are embryonic lethal and have defects in epithelial integrity (Tepass et al. 1996; Uemura et al. 1996). DE-cadherin has seven cadherin domains, a cysteine-rich proteolytic region, an EGF-like region, and a laminin globular domain followed by the transmembrane domain and the cytoplasmic tail (Oda and Tsukita 1999). During post-translational modification, DEcadherin is glycosylated (Oda et al. 1993; Peifer 1993) and its extracellular region is cleaved at its cysteine-rich region which then re-associates in the mature protein via a cystine disulfite bond (Oda and Tsukita 1999). The cytoplasmic tail of E-cadherin binds p120catenin at the juxtamembrane position (Myster et al. 2003) and Armadillo via forty-one amino acids near its C-terminus (Pai et al. 1996).

p120catenin contains 10 armadillo repeats in its central region. The first two and the last two armadillo repeats are necessary for DE-cadherin binding. Null mutants of p120catenin are viable and fertile. However, loss of p120catenin enhances the mutant phenotype of shg mutants
suggesting a supportive role of p120catenin in stabilizing adherens junctions and retaining adherens junction molecules at the plasma membrane. Similarly, mammalian p120catenin interferes with E-cadherin endocytosis (Myster et al. 2003).

Armadillo has 13 armadillo repeats, an acidic N-terminal and a Gly-Pro rich region at its C-terminus (Peifer and Wieschaus, 1990). Armadillo repeats 3-8 are necessary for binding a serine-rich region in the cytoplasmic tail of DE-cadherin. The first armadillo repeat and the N-terminal region is required for the binding to α-Catenin (Orsulic and Peifer 1996). Armadillo is homologous to β-catenin and plakoglobin in vertebrates. Armadillo binds DE-cadherin in the endoplasmic reticulum and is important for its effective transportation to the adherens junctions (Chen et al., 1999; Lock et al., 2005; Langevin et al., 2005). Armadillo is also important to give structure to the cytoplasmic tail of DE-cadherin (Huber et al., 2001). Maternal zygotic mutant of Armadillo are embryonically lethal and show a severe loss of epithelial integrity during embryogenesis (Cox et al., 1996).

Similar to its mammalian counterpart, Drosophila α-Catenin has three vinculin homology domains: VH1, VH2 and VH3 (Oda et al., 1993). Monomeric α-Catenin links the cadherin-β-catenin complex to the actin cytoskeleton and is presumably in a dynamic equilibrium with α-Catenin dimers in the cytoplasm (Desai et al., 2013). α-Catenin is also known to have mechanosensing properties. As tension is applied at adherens junctions, α-Catenin changes conformation revealing cryptic bindings sites for actin binding proteins such as vinculin, which can then bind and strengthen the bond between adherens junctions and the actin cytoskeleton to withstand force (Yonemura et al., 2010; le Duc et al., 2010; Yonemura 2011, Twiss et al., 2012; Kim et al., 2015). α-Catenin is the core component of the cadherin-catenin complex that
mediates the interaction between E-cadherin and the actin cytoskeleton (Kwiatkowski et al., 2010, Harris and Tepass, 2010; Harris, 2012; Maiden and Hardin, 2011; Leckband and de Rooji, 2014). The component of the CCC that my project was focused on was Armadillo/β-catenin and its function at adherens junctions.
Figure 1. Components of the junctional complexes in *Drosophila*. (A) In *Drosophila*, junctional complexes are composed of septate junctions and adherens junctions (Zonula adherens). (B) Molecular composition of adherens junctions. (C) Cadherin-catenin complex components and their domain organization (adopted from Harris, 2012).
1.2.3 Formation and Maturation of Adherens Junctions

This section focuses on the formation and maturation of adherens junctions in *Drosophila*. After fertilization, the zygote undergoes 13 nuclear divisions forming a syncytium of ~5000 nuclei around the embryo surface. Then the plasma membrane from the embryo surface invaginates to compartmentalize nuclei synchronously into ~5000 individual cells. This process of cellularization results in the formation of an epithelium of columnar hexagonal cells, the blastoderm. Adherens junctions are first formed during cellularization. In strong mutants of adherens junction components, the blastoderm still forms suggesting that cellularization does not require adherens junctions (Cox *et al.* 1996; Harris and Peifer 2004; Sokac and Wieschaus 2008).

The apical surface of each cell is covered in actin-based protrusions in which cadherin-catenin clusters are first assembled (McGill *et al.* 2009). As cellularization proceeds, autocellular clusters between protrusions of the same cell are gradually removed while intercellular clusters mature into larger structures called spot adherens junctions (Tepass and Hartenstein 1994; McGill *et al.* 2009). Spot adherens junctions form around the circumference of cells at an apicolateral position where centrosomes are found. An average spot adherens junction consists of 1500-1800 molecules of DE-cadherin and Armadillo in a 1:1 stoichiometry ratio (McGill *et al.* 2009).

Bazooka, the *Drosophila* homologue of Par-3, is a polarity protein needed to assemble spot adherens junctions in the apico-lateral membrane. In Bazooka deficient embryos, the cadherin-catenin complex fails to concentrate and form into spot adherens junctions (Harris and Peifer 2004; McGill *et al.* 2009). Bazooka is found also in clusters around the apical circumference at the same apical-basal position as centrosomes and colocalize with adherens junction clusters (Harris and Peifer 2004). Bazooka clusters form even in the absence of adherens junctions,
suggesting that it works upstream of adherens junction clustering. The minus-end directed motor Dynein and an actin-based apical scaffold are important to position the Bazooka clusters (Harris and Peifer 2005). Once established in the apico-lateral membrane Bazooka is required to concentrate cadherin-catenin clusters that formed between apical protrusions to form spot adherens junctions. In both Canoe and Rap1 mutants, Bazooka looses its apical enrichment suggesting that Canoe and small GTPase Rap1 act upstream of Bazooka and are essential for the initial apical positioning of Bazooka and adherens junctions (Choi et al. 2013). However, Atypical Protein Kinase C (aPKC) and Bazooka are required for Canoe localization during cellularization suggesting that early polarity maintenance involves a network of protein interactions rather than a linear pathway (Choi et al. 2013).

After cellularization, the embryo undergoes gastrulation in which spot adherens junctions are converted into belt-like zonulæ adherentes (Stage 7-8, Tepass and Hartenstein 1994). During this conversion, adherens junctions gain a greater localization with actin and lose their association with microtubules and move away from centrosomes to a more extreme apical position (Harris and Peifer 2004). aPKC is important for transitioning adherens junctions from their earlier microtubule-based localization into a more belt-like adherens junctions around the apical circumference (Harris and Peifer, 2007). Once adherens junctions gain association with actin, Crumbs and Bitesize, a synaptotagmin-like protein are both important to form a complex with Moesin and modify the apical cytoskeletal network to promote the formation of belt-like adherens junctions (Tepass 1996; Pilot et al. 2006). α-Catenin is also important for connecting the cadherin-catenin complex to the apical actin network (Cavey et al. 2008; Martin et al. 2010). The formation and maintenance of the circumferential adherens junctions or zonula adherens requires apical and basolateral polarity protein complexes. There are two major apical
complexes, the Crumbs and Par complexes, and two major basolateral complexes, the lethal giant larvae (Lgl) and Yurt (Yrt)/Coracle (Cora) groups. These apical and basolateral, as well as junctional complexes, functionally define distinct polarized membrane domains in epithelial cells (Laprise and Tepass 2011; Tepass 2012).

1.3 Structure and Function of β-catenin

β-catenin is a 781 amino acid protein that contains 12 α-helical Armadillo repeats (Figure 2). Each Armadillo repeat is formed from three α-helices arranged in a triangular shape. Together, the Armadillo repeats form a superhelix that has a positively charged groove. There is a 71% sequence similarity between Armadillo and β-catenin. β-catenin is involved in adhesion as part of the cadherin-catenin complex, but a junction-independent cytoplasmic pool of β-catenin also functions in Wnt signalling (Huber et al. 1997; Nusse 1997; Clevers and Nusse 2012; Valenta et al. 2012). In the cadherin-catenin complex, β-catenin/Armadillo acts as a scaffold between E-cadherin and α-Catenin. Several reports have suggested that Armadillo plays a regulatory role in adherens junctions assembly and stability apart from behaving as a physical linker between DE-cadherin and α-Catenin (Gorfinkiel and Arias 2007; Tamada et al. 2012; Langevin et al. 2005; Mirkovic et al. 2011; Petzoldt et al. 2012). Whether β-catenin serves only as a linker between DE-cadherin and α-Catenin, or in addition acts to regulate adherens junction stability may differ between tissues.
Figure 2. Structure and binding partners of β-catenin. β-catenin is a 781 amino acid protein that contains 12 α-helical Armadillo (Arm) repeats. Arm repeats 3 to 8 are necessary and sufficient for DE-cadherin binding (shown in green) while α-Catenin binds to the junction of the N-terminal region and the start of the Arm repeats (shown in orange). Blue boxes show kinases known to phosphorylate Armadillo at certain phosphorylation sites. Schematic was created using the Simple Modular Architecture Research Tool.

1.3.1 β-catenin functions as a nuclear transcription factor in Wnt Signaling

β-catenin/Armadillo functions as a nuclear transcription factor in the Wnt signaling pathway. The Wnt/Wg pathway is required for many different developmental processes including establishing segmental polarity in Drosophila embryos and adults. Mutants of Armadillo or wingless (wg) display segment polarity defects in the larval cuticle, in which a lawn of disoriented denticles are observed instead of regular rows of denticles interspersed with naked cuticle. Armadillo’s role in adhesion is independent of its role in Wg signaling. There are two pools of Armadillo, one at the adherens junctions and one in the cytoplasm responsible for Wg signalling. In the absence of Wg signaling, levels of cytoplasmic Armadillo are kept low by phosphorylation by protein kinase Zw-3 (GSK-3β). Therefore, in the absence of Wg signaling, most of the Armadillo is located at adherens junctions for its function in adhesion. However, in the presence of a Wg/Wnt signalling, Wg/Wnt binds to its receptor (Dfz2 or other members of
the Frizzled family), activating Dishevelled (Dsh) to inactivate Zw-3/GSK-3b, preventing it from phosphorylating Armadillo and increasing its cytoplasmic level. Once Armadillo levels rise in the cytoplasm, they bind to the transcription factor TCF1/Lef-1/pangolin and travel to the nucleus where this complex activates nuclear target genes, such as Ubx, a homobox gene (Riese et al. 1997) or wingless (Yu et al. 1998).

1.3.2 β-catenin acts primarily as a scaffold between DE-cadherin and α-Catenin

In the Drosophila ovary, the primary function of Armadillo is to physically link DE-cadherin and α-Catenin. Epithelial tissue integrity, border cell migration, and oocyte positioning defects of Armadillo mutants have been rescued by the expression of a DEcadherin::αCatenin fusion protein which directly link DEcadherin to α-Catenin. Border cell migration and oocyte positioning are both dynamic processes that require regulation of adherens junctions. These results suggest that the primary function of Armadillo at adherens junctions is to link DE-cadherin and α-Catenin. Any regulatory role that Armadillo may have is not essential for tissue and cells in which expression of DEcadherin::αCatenin can compensate for the loss of the endogenous cadherin-catenin complex (Sarpal et al. 2012; Pacquelet and Rørth 2005).

1.3.3 β-catenin is important for regulating adherens junctions

Several studies have proposed that Armadillo at adherens junctions is important for the regulation of adherens junction stability in addition to its essential role in linking DE-cadherin to α-Catenin. These studies focused on Armadillo’s function in dorsal closure, germ band extension, trafficking of DE-cadherin to the membrane, and genitalia and ommatidial rotation (Gorfinkiel and Arias 2007; Tamada et al. 2012; Langevin et al. 2005; Mirkovic et al. 2011; Speder et al. 2006).
Armadillo plays an essential role in addition to its function in linking DE-cadherin to α-Catenin at adherens junctions during dorsal closure (Gorfinkiel and Arias, 2007). These authors presented evidence suggesting that shotgun mutants that lack 61 amino acids from the cytoplasmic tail of DE-cadherin, which includes the β-catenin binding site, exhibit dorsal holes that can be rescued by the expression of DEcad::αCat in epidermal stripes or in the amnioserosa. However, a fusion protein lacking the Armadillo binding site, DEcadΔ61::αCat does not rescue the dorsal defects in shotgun mutant embryos. Additionally, DEcad::αCat cannot rescue dorsal closure defects in Armadillo mutant embryos. This suggests that the lack of Armadillo at adherens junctions prevented normal dorsal closure and that Armadillo has a role in dorsal closure that is separate from its role as a linker between DE-cadherin to α-Catenin.

Phosphorylation of β-catenin on tyrosine 142 by Fyn, Fer and cMet kinases reduce its affinity for α-Catenin (Ozawa and Kemler 1998; Piedra et al. 2003; Brembeck et al. 2004; Tominaga et al. 2008) whereas phosphorylation of β-catenin on tyrosine 654 (Y654) reduces its affinity for E-cadherin (Roura et al. 1998; Bonvini et al. 2001; Piedra et al. 2001; van Veelan et al. 2011).

Phosphorylation of β-catenin, particularly at Y654, has been shown to regulate cadherin activity in mammalian cell culture studies (Lilien and Balsamo 2005). When Y654 is phosphorylated, β-catenin loses its affinity for cadherin, resulting in a decrease in cell adhesion. Y667 in Armadillo is homologous to Y654 in mammalian β-catenin. Phosphomutant ArmadilloY667F can rescue epithelial integrity in all cadherin-mediated processes in the Drosophila ovary, suggesting that phosphorylation of Y667 is not required during oogenesis (Pacquelet and Rorth 2005). Therefore, regulation of adherens junctions must not be occurring through Armadillo by phosphorylation at this site, consistent with the finding that DEcadΔ61::αCat can rescue shotgun and Armadillo mutant defects in the ovary (Pacquelet and Rorth 2005).
In contrast to oogenesis, phosphorylation of Armadillo at Y667 by Abelson tyrosine kinase is important for adherens junction dynamics and cell rearrangement during germ band extension in embryonic epithelial cells, in a process that drives cell intercalation during germ band elongation (Tamada et al. 2012). Time-lapse imaging experiments show that non-phosphorylatable Armadillo mutant recapitulates defects associated with Abelson mutants, such as failing to mediate rosette formation and have delayed axis elongation. Whereas a phosphomimetic form of Armadillo increase the turnover of Armadillo at adherens junctions and rescue all the defects associated with rosette formation in Abelson-deficient embryos (Tamada et al. 2012). Together these results suggest that Abelson kinase phosphorylates Armadillo at Y667 and is important for Armadillo turnover, which promotes cell rearrangement that drives axis elongation.

In the cells of the notum of the Drosophila wing imaginal disc, Armadillo was shown to be important for the delivery of DE-cadherin to the plasma membrane by interacting with the exocyst complex (Langevin et al. 2005). Using antibody binding uptake experiments, it was found that Sec5 is important for the transcytosis of basolateral DE-cadherin to the apical adherens junctions. Loss of Sec5, Sec6 and Sec15 function is associated with accumulation of DE-cadherin in enlarged recycling endosomes similar to Armadillo mutants. Sec10 can physically interact with Armadillo suggesting that Armadillo can exist in a complex with exocyst components. Together these results suggest that Armadillo provides a landmark in order to deliver DE-cadherin from the recycling endosomes to the adherens junctions.

Ommatidial rotation in the Drosophila eye is a DE-cadherin-dependent cell-motility process in which photoreceptor preclusters rotate from their original anterior-posterior axis towards the dorsal-ventral axis to form the proper photoreceptor arrangement in each ommatidium (Mirkovic
et al. 2011; Mirkovic and Mlodzik 2006). Nemo-kinase - dependent serine-threonine phosphorylation of Armadillo is important for promoting ommatidial rotation, even though it is not important for the formation of Armadillo-DE-cadherin complex in vitro (Mirkovic et al. 2011). DE-cadherin and α-Catenin fusion proteins do not rescue the loss of Nemo activity, suggesting that Armadillo phosphorylation by Nemo is modulating its interaction with other binding partners such as α-Catenin, a regulatory step required for ommatidial rotation (Mirkovic et al. 2011).

Unconventional type ID myosin (MyoID) acts as a left-right determinant responsible for the clockwise (dextral) rotation of genitalia in Drosophila male flies by physically interacting with Armadillo (Speder et al. 2006). The MyoID tail domain binds Armadillo in vitro. In vivo, both MyoID and Armadillo colocalize at the adherens junctions in the A8 segment of the male genital disc, the left-right organizer for genitalia. This interaction is important to promote MyoID activity for establishing left-right asymmetry, while inhibiting the activity of a second myosin, MyoIC (Petzoldt et al. 2012; Speder et al. 2006)

Taken together, these studies suggest a role of Armadillo in cell-cell adhesion aside from simply linking DE-cadherin to α-Catenin. However, these additional functions of Armadillo seem to be highly tissue specific. To further clarify the function of Armadillo in morphogenesis, I have re-examined its role in dorsal closure and also studied its function in the development of the ventral epidermis.
1.4. Ventral Epidermis

During early embryogenesis (3h15-4.5h AEL), the ventral ectoderm undergoes three main morphogenetic processes that will lay out the body plan of the embryo: convergence-extension to elongate the anterior-posterior axis, ingression of neural progenitor cells (neuroblasts), and cell division in predefined cellular clusters across the ectoderm (Figure 3).

At stage 7, ~2600 lateral cells from the ventral ectoderm (the germband) extend by means of convergent extension to elongate the body axis of the developing embryo in the anterior-posterior direction. Cell intercalation (T1 processes) and rosette formation are the driving forces of germ band extension (Irvine et al. 1994; Bertet et al. 2004; Blankenship et al. 2006; Tamada et al. 2012). During cell intercalation, single vertical cell contacts constrict and are lost, joining cells previously separated along the dorso-ventral axis through a new horizontal cell-cell contact. During rosette formation, multiple linked vertical cell-cell contacts constrict to a vertex and resolve perpendicularly along the anterior-posterior axis resulting in 5-11 cells changing neighbors and extending the germband. During germband extension, cells are planar polarized such that adherens junction proteins are enriched at the dorsal-ventral contacts while actin-myosin networks are enriched along the anterior-posterior contacts (Bertet et al. 2004; Blankenship et al. 2006; Zallen and Wieschaus 2004; Harris 2012; Wu and Yap 2013; Takeichi 2014; Lan et al. 2014). Actin-myosin network also exists at the apical surface and undergoes repeated pulsed constrictions that coincide with the constriction of the anterior-posterior (vertical) contacts (Rauzi et al. 2010). The actomyosin network at the anterior-posterior contacts act as a ratchet to maintain the constricted state leading to oriented cell movements (Rauzi et al. 2010). Rosettes are formed when constriction along one anterior-posterior contact leads to the assembly of actomyosin network in cells above and below. This forms a supracellular
actomyosin cable that runs down multiple cell contacts which then contracts to form rosettes (Fernandez-Gonzalez et al. 2009). As anterior-posterior contacts constrict, adherens junctions are removed from these shrinking edges through planar-polarized endocytosis (Levayer et al. 2011) and exclusion of Bazooka/Par-3, an upstream regulator of the Cadherin-Catenin complex, by the myosin regulator Rho-kinase (Simões et al., 2010). Once anterior-posterior contacts have constricted and new contacts form, adherens junctions are required at these newly formed dorsal-ventral contacts to maintain epithelial integrity. The dorsal-ventral contacts first accumulate F-actin and DE-cadherin and then Bazooka/Par3 after a short delay (Blankenship et al., 2006).

Concurrent with germband extension, neural progenitor cells in the ventral ectoderm (neuroectoderm) undergo epithelial-mesenchymal transitions by which they ingress to become neural stem cells or neuroblasts. These neuroblasts eventually give rise to the Drosophila central nervous system (Hartenstein and Wodarz 2013). Notch-delta signaling separates neural cells from ectodermal cells through lateral inhibition. Ingressing cells must lose cell-cell contacts with neighbours and once delaminated, the neighboring cells need to re-establish cell-cell contacts in order to avoid epithelial discontinuity. When all epithelial cells are forced to become neuroblast cells, as in Notch/Delta mutants, adherens junctions and epithelial integrity are lost in the neurectoderm, suggesting that new junctions formed by neighboring, non-neural cells, during neuroblast ingestion are critical to maintain epithelial stability (Lehmann et al. 1983). Accordingly, embryos that do not express DE-cadherin zygotically and thus, are unable to form new stable junctions during morphogenesis, show lack of epithelial integrity in the neuroectoderm (Tepass et al. 1996).
As the germband extends, groups of cells (mitotic domains) begin to divide in the ventral ectoderm. Adherens junctions have to resolve and reform as cells divide in the plane of the epithelium (Foe et al. 1993; Harris and Peifer 2004; Lu et al. 2001; Cavey and Lecuit 2009; Founounou et al. 2013; Herszterg et al. 2013; Guillot and Lecuit 2013; Bourdages and Maddox 2013). As cells undergo mitotic division, they round up and have an enlarged circumference to accommodate the mitotic spindle. While this happens, adherens junctions show a diminished electron-dense undercoat (Tepass and Hartenstein 1994). However, once cells divide, Canoe is thought to be important to reassemble adherens junctions between the daughter cells to maintain them within the epithelial sheet (Sawyer et al., 2009). A recent paper from the Lecuit lab reports that during epithelial cytokinesis, the cleavage that occurs along the apico-basal axis is polarized such that it invaginates faster basally than apically. However, this polarized cleavage is not due to polarized contraction of the actomyosin ring but rather due to the ring being apically anchored to adherens junctions. They also found that cleavage occurs in the plane of adherens junctions and requires local adhesion disengagement. This disengagement is mechanically induced when the tension in the cytokinetic ring and the tension exerted through intercellular adhesion by neighbouring cells is higher than the adhesive force. Once disengagement occurs, there is new E-cadherin complex formation at the new interface, allowing cells to preserve tissue integrity while undergoing division (Guillot and Lecuit, 2013).

These processes make the ventral epidermis a highly morphogenetically active tissue that is sensitive to adherens junction disruption therefore making it a good model to study the regulation of cell adhesion during morphogenesis.
Figure 3. Morphogenetic movements in the ventral epidermis. The ventral ectoderm undergoes three main morphogenetic processes: (A) ingress of neural progenitor cells (neuroblasts), (B) cell division and (C) convergence-extension to elongate the anterior-posterior axis.
1.5 Dorsal Closure

*Drosophila* dorsal closure is a process by which lateral epithelia converge dorsally to cover the dorsal aspect of the embryo, while the extraembryonic amnioserosa is internalized (Figure 4). Dorsal closure begins after germ band retraction (10h AEL), and the initial cue is believed to be an up-regulation of JNK signalling at the leading edge (Thomas *et al.* 2009). By stage 13, a supra-cellular actin cable has formed at the dorsal edge of the leading edge cells. Echinoid is expressed in the ectoderm but not in the amnioserosa and this differential expression acts as a cue to organize an actin cable at the boundary between these tissues (Laplante and Nilson 2006). Two coordinated cell shape changes drive dorsal closure; the dorso-ventral elongation of epidermal cells and the pulsed apical constriction of amnioserosa cells. The pulsed apical constriction is driven by contractile actin-myosin networks that flow across the apical surface of the amnioserosa cells (Blanchard *et al.* 2010; David *et al.* 2010). Apical actin and myosin accumulation begins as early as germ band extension and eventually by the time the germ band retracts, all cells show these dynamic actin-myosin contractions (David *et al.* 2010). Thus, the mechanism by which the cells undergo apical constriction involves myosin motors binding and sliding along an actin network spanning the medial apical cortex which connects to the cell membrane through the adherens junctions (Blanchard *et al.* 2010; David *et al.* 2010), similar as in during the invagination of the mesoderm in early embryos (Martin *et al.* 2010; Martin *et al.* 2009). It was also proposed that there is a progressive enrichment of myosin at the apical surface that acts as a ratchet to prevent relaxation once cells undergo constriction (Martin *et al.* 2009). As dorsal closure progresses, amnioserosa fluctuations decrease in amplitude and the cycle length of each fluctuation decreases, while increasing the rate of contraction (Blanchard *et al.* 2010, David *et al.* 2010). The amnioserosa also undergoes basal extrusion of cells in a random
spatial pattern followed by programmed cell death (Fernández et al. 2007; Kiehart et al. 2000; Toyama et al. 2008). At stage 14, lamellipodia and filopodia protrude from the leading edge cells to contact each other at the posterior and anterior-most ends where epidermal cells from opposite sides of the embryo are close enough to interdigitate and zip together. These leading edge cells take on in part mesenchymal properties, including loss of apical and basal polarity complexes and septate junctions at the edge where cell protrusions form (Bahri et al. 2010). Adherens junction components are also present at lower levels in filopodia (Gorfinkiel and Arias 2007). The C-terminus of DE-cadherin interacts with a Myosin XV homolog Sisyphus and is trafficked to the filopodia by this interaction (Liu et al., 2008). Once the filopodia interdigitate, adherens junction proteins become enriched at those sites (Jacinto et al. 2000). By stage 15, a midline seam has formed and the process of dorsal closure is completed (Kiehart et al. 2000; Jacinto et al. 2002; Solon et al. 2009; David et al. 2010; Blanchard et al. 2010; Laplante and Nilson 2011; Gorfinkiel et al. 2011; Harris 2012). Adherens junctions are also found connecting epidermal cells and amnioserosa cells at the interface between these tissues. The contacts between the epidermis and amnioserosa must regulate adhesion to withstand forces associated with the process of dorsal closure. Insufficient adhesion can lead to failure of dorsal closure making it another excellent model to study cell-cell adhesion during morphogenesis (Kiehart et al. 2000; Jacinto et al. 2002; Solon et al. 2009; David et al. 2010; Blanchard et al. 2010; Laplante and Nilson 2011; Gorfinkiel et al. 2011; Harris 2012).
Figure 4. The process of dorsal closure. *Drosophila* dorsal closure begins at stage 12, it’s a process by which lateral epithelia converge dorsally to cover the dorsal aspect of the embryo, while the extraembryonic amnioserosa is internalized. By stage 13, a supra-cellular actin cable forms at the apical edge of the LE cells. Two coordinated cell shape changes drive DC; the dorso-ventral elongation of epithelial cells and the pulsed apical constriction of amnioserosa cells. At stage 14, lamellipodia and filopodia protrude from the LE cells to contact each other at the posterior and anterior-most ends where epithelia are close enough to interdigitate and zip together. By stage 15, a midline seam is formed where these two epithelia meet and the process of dorsal closure is completed. This diagram is adapted from Woolner and Martin (2006).

1.6 Thesis Objective

β-catenin is a multifunctional protein, which makes it difficult to separate its functions in development as a nuclear effector of Wnt signalling and as a scaffold between E-cadherin and α-Catenin at adherens junctions. From previous studies that have looked into the role of β-catenin specifically at adherens junctions, it remains controversial whether β-catenin acts simply as a linker between DE-cadherin and α-Catenin or whether it has other regulatory roles essential for adherens junction function. The goal of my thesis was to examine the function of β-catenin at adherens junctions during dorsal closure and the formation of the ventral epidermis. In order to
study its function, I attempted to rescue defects in dorsal closure and ventral epidermis in *shotgun* and *armadillo* mutant embryos using fusion proteins that directly link DE-cadherin to α-Catenin and lack the Armadillo binding site. If fusion proteins that prevent β-catenin localization at adherens junctions can rescue dorsal closure and formation of the ventral epidermis, it would suggest that the β-catenin is acting primarily as a linker between DE-cadherin to α-Catenin. However, inability of the fusion proteins to elicit a rescue would suggest that β-catenin has other regulatory roles that are essential for these morphogenetic events (Figure 5).

**Figure 5. Model of Fusion Proteins.** (A) In wildtype, β-catenin links DE-cadherin to α-Catenin. Apart from its linker function, β-catenin can also perform regulatory functions required for AJ assembly and stability. For example, it can be phosphorylated by various kinases to allow for disassembly of adherens junctions or be involved in recruiting adherens junctions components to the membrane. (B) When the DEcad::αCat fusion protein is expressed in DEcadherin deficient embryos, β-catenin can still bind to this fusion protein since DE-cadherin and α-Catenin both retain their β-catenin binding sites. Therefore, β-catenin is still available at the cadherin-catenin complex to perform additional regulatory function required for AJ stability apart from being a linker. (C) By expressing fusion proteins that directly link DE-cadherin to α-Catenin and lack the β-catenin binding site of both DE-cadherin and α-Catenin in DE-cadherin deficient embryos, it prevents β-catenin localization at adherens junctions. β-catenin is no longer found at the cadherin-catenin complex to perform any function. If such fusion proteins rescue, it would suggest that β-catenin has no other role apart from linking DE-cadherin to α-Catenin.
Chapter 2
Materials and Methods

2.1 Drosophila Stocks

The following table shows the fly stocks used for the rescue experiments:

Table 1. List of fly stocks used for shg$^{R69}$ and arm$^4$ rescue experiments.

<table>
<thead>
<tr>
<th>Fly Stocks</th>
<th>Full Genotype</th>
<th>Source of Stock</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEcad</td>
<td>UAS-DEcad</td>
<td>Ritu Sarpal (University of Toronto)</td>
<td>(Sarpal et al. 2012)</td>
</tr>
<tr>
<td>DEcad::αCat</td>
<td>UAS- DEcad::αCat</td>
<td>Ritu Sarpal (University of Toronto)</td>
<td>(Sarpal et al. 2012)</td>
</tr>
<tr>
<td>DEcadΔ61::αCat</td>
<td>UAS- DEcadΔ61::αCat/ TM3-ser</td>
<td>Pernille Rorth (University of Copenhagen)</td>
<td>(Pacquelet and Rørth 2005)</td>
</tr>
<tr>
<td>DEcadΔβ::αCatΔVH1</td>
<td>UAS- DEcadΔβ::αCatΔVH1</td>
<td>Ritu Sarpal (University of Toronto)</td>
<td>-</td>
</tr>
<tr>
<td>en-GAL4</td>
<td>y¹ w* ; P{en2.4-GAL4}e16E/CyO</td>
<td>Bloomington Drosophila Stock Centre</td>
<td>(Fietz et al. 1995)</td>
</tr>
<tr>
<td>da-Gal4 (III)</td>
<td>w* ; P{GAL4-da.G32}UH1</td>
<td>Bloomington Drosophila Stock Centre</td>
<td>(Wodarz et al. 1995)</td>
</tr>
<tr>
<td>mat67-Gal4</td>
<td>P{matα4-GAL-VP16}; Dr/TM6b</td>
<td>Daniel St. Johnson (University of Cambridge)</td>
<td>(Hacker and Perrimon 1998)</td>
</tr>
<tr>
<td>shg$^{R69}$</td>
<td>FRT42D shg[R69b] / SM6B, cn</td>
<td>Ulrich Tepass (University of Toronto)</td>
<td>(Godt and Tepass 1998)</td>
</tr>
<tr>
<td>arm$^4$</td>
<td>y¹ arm4 w*/FM7c</td>
<td>Bloomington Drosophila Stock Centre</td>
<td>(Peifer and Wieschaus 1990)</td>
</tr>
</tbody>
</table>
All the stocks used are in the white background. The following lines containing Gal4 driver were used to express the transgenes: en-Gal4 (Johnson et al., 1995), da-Gal4 (Wodarz et al. 1995), and mat67-Gal4, a gift from D. St. Johnston & F. Wirtz-Peitz.

The following table shows the UAS-transgenes used for the rescue experiments:

**Table 2. List of UAS-transgenes used for shgR69 and arm4 rescue experiments.**

<table>
<thead>
<tr>
<th>Constructs</th>
<th>Amino Acid Present</th>
<th>Insertion Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEcad</td>
<td>1-1507</td>
<td>attP2</td>
</tr>
<tr>
<td>DEcad::αCat</td>
<td>1-1507/1-917</td>
<td>attP2</td>
</tr>
<tr>
<td>DEcadΔ61:: αCat</td>
<td>1-1446/1-917</td>
<td>Randomly inserted into 3rd Chromosome</td>
</tr>
<tr>
<td>DEcadΔβ:: αCatΔVH1</td>
<td>1-1445+1445-1466/281-917</td>
<td>attP2</td>
</tr>
</tbody>
</table>

The following recombinant lines were generated for embryonic rescue experiments:

1. en-Gal4, shgR69/CyO-twi-Gal4, UAS-GFP
2. mat67-Gal4, shgR69/CyO-twi-Gal4, UAS-GFP

The following are driver lines created for embryonic rescue experiments as well as the no driver control:

1. shgR69/CyO-twi-Gal4, UAS-GFP; da-Gal4
2. shgR69/CyO-twi-Gal4, UAS-GFP

The following lines carrying each of the transgenes (Table 2) were crossed to a line with an appropriate driver. These lines were recombined over a fused balancer of chromosome 2 and 3.

1. shgR69, DEcad/ CyO-TM6b
2. shgR69, DEcad::αCat/ CyO-TM6b
3. \textit{shg}^{R69},\textit{DEcad}^{Δ61}::\textit{αCat}/\textit{CyO-TM6b}

4. \textit{shg}^{R69},\textit{DEcad}^{Δβ}::\textit{αCat}^{ΔVH1}/\textit{CyO-TM6b}

The crosses that were made were (\textit{UAS-X} stands for all the transgenes listed in Table 1):

1. \textit{mat67-Gal4, shg}^{R69}/+; \textit{UAS-X}/+ x \textit{shg}^{R69}/\textit{CyO-twi-Gal4, UAS-GFP; da-Gal4}

2. \textit{shg}^{R69}/\textit{CyO-twi-Gal4, UAS-GFP; da-Gal4} x \textit{shg}^{R69},\textit{UAS-X}/\textit{CyO-TM6b}


4. \textit{shg}^{R69}/\textit{CyO-twi-Gal4, UAS-GFP} x \textit{shg}^{R69},\textit{UAS-X}/\textit{CyO-TM6b}

\textbf{2.2 Rescue Experiments}

For evaluating the ability of transgenes to rescue \textit{shg}^{R69} mutant defects, the transgenes listed in Table 2 were expressed with different drivers in the \textit{shg}^{R69} mutant background. The eggs were collected on apple juice agar plates at 25°C. 100-200 fertilized non-GFP embryos were collected allowed to develop at 25°C. Those that did not hatch were collected, dechorionated using 50% bleach for 4 minutes, then washed in PBST, and mounted in Hoyer’s medium and lactic acid (1:1 ratio) on a slide. Slides were left in 85°C overnight to clear the embryos for microscopic examination of the larval cuticle.

\textbf{2.2.1 Scoring Cuticle Preparations}

The cuticle preparations from the rescue crosses were assessed for dorsal and ventral defects and were given the following scoring numbers- Ventral defects were scored based on the number of intact denticle belts (0-8). A score of (0) meant an intact ventral cuticle whereas a score of (8) would mean the entire ventral cuticle is defective or absent.
Dorsal holes were given scores based on whether there was an intact dorsal cuticle (no defect-0), a defect was apparent in the cuticle but no hole was visible (minor defect-1), a dorsal hole that is small enough that it is retained within one or two segments (small hole-2), a hole that extends beyond two segments (partial hole-3) or a hole that starts from the head and ends at the spiracles (complete hole-4).

2.2.2. Statistical Analysis

Data were represented as mean ± standard deviation (s.d.). Statistical significance was assessed using two-tailed unpaired Student’s t-test with a 99% confidence interval in GraphPad Prism software.

2.3 Lethality Counts

For overexpression analysis, homozygous lines of each transgene listed in Table 2 were crossed to da-GAL. The eggs were collected on apple juice agar plates at 25°C. 100-200 fertilized embryos were collected and allowed to develop at 25°C. Hatched, larvae were subjected to lethality counts at each of the subsequent stages of Drosophila development: embryo, first instar, second instar, third instar, pupae, and adult. The lethality count at each developmental stage was divided over n total number of eggs collected and converted to a percentage of lethality. The data were then represented as the mean ± standard deviation (s.d.) of percent lethality at each developmental stage.

2.4 Immunocytochemistry

For the localization of constructs at the membrane, transgenes listed in Table 2 were crossed to en-Gal4 driver in a shg^{R69} mutant background. Drosophila embryos were dechorionated in 50%
bleach for 4 minutes. Then, the embryos were fixed in 5% formaldehyde for 20 minutes.

Standard antibody staining protocols were used to label fixed embryos with antibodies. Embryos were blocked with 2% NGS. The primary antibodies used were: anti-DCAD2 (rat monoclonal, 1:50; Developmental Studies Hybridoma Bank (DSHB)) and anti-Arm (mouse monoclonal, 1:50; DSHB). Secondary antibodies were conjugated to either, Alexa Fluor- 555 and Alexa Fluor – 647 or to Cy3 or Cy5 (Jackson Immunoresearch Laboratories). Stained embryos and ovaries were mounted in Vectashield (Vector Labs). The samples were imaged with a Leica SP8 confocal microscope using 63x NA 1.4 oil immersion lens.

2.5 Quantitative Immunoblots

For immunoblotting of lysates derived from embryos that overexpressed the transgenes listed in Table 2, each transgene was crossed to da-Gal4. *Drosophila* embryos collected overnight were dechorionated in 50% bleach for 4 minutes. Subsequently, embryos were washed in PBST and homogenized in Lamelli Buffer (950 μL of 2x Biorad® Laemmli Sample Buffer, 50 μL of 2-Mercaptoethanol). Protein concentrations were measured using standard Trichloroacetic acid (TCA) precipitation followed by spectrophotometry. 60 μg of lysate from whole embryos were loaded into each well. Proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane following the instructions provided by the Life Technologies iBlot® 2 Dry Blotting System. Life Technologies iBind™ Western System was used to detect the proteins on the nitrocellulose membrane. The primary antibodies used for detection of proteins were anti-DEcad (Rat monoclonal DCAD1, 1:500, ; DSHB), anti-α-Cat (Guinea pig polyclonal-121, 1:1000; Sarpal et al. 2012), anti-tubulin (Mouse monoclonal E7, 1:500; DSHB). The secondary antibodies used for visualization were: anti-Rat 680LT (Goat, 1:5000; Licor), anti-mouse 680LT
(Donkey, 1:5000; Licor), and anti- Guinean Pig 800CW (Donkey, 1:5000; Licor). The proteins were visualized using the LI-COR Odyssey® Fc Dual-Mode Imaging System.
Chapter 3

Results

To ask whether β-catenin/Armadillo at adherens junctions is dispensable during dorsal closure and in the ventral epidermis I attempted to rescue dorsal and ventral cuticle defects in \textit{shg}^{R69} (null allele) and \textit{arm}^{4} (null allele) mutant embryos using fusion proteins: DEcad, DEcad::αCat, DEcadΔ61::αCat (Pacquelet and Rørth 2005; Gorfinkiel and Arias 2007), and DEcadΔβ::αCatΔVH1 (Figure 5). The drivers I used to overexpress these trangenes were the maternal driver mat67-Gal4, daughterless-Gal4 (da-Gal4) (ubiquitous driver), en-Gal4 (segmental stripe driver) used previously by Gorfinkiel and Arias (2007), and a no driver control.

Part 1- Fusion proteins lacking the Armadillo binding site do not localize Armadillo to adherens junctions

In contrast to DEcad and DEcad::αCat in which full-length DE-cadherin is fused to full length α-Catenin, DEcadΔ61::αCat and DEcadΔβ::αCatΔVH1 lack the Armadillo binding sites (Figure 5). The DEcadΔ61::αCat fusion protein (Pacquelet and Rørth 2005) carries a deletion of the last 61 amino acids of the DEcad cytoplasmic tail that removes the Armadillo binding site but may also compromise other molecular interactions of DEcad such as interactions with Myosin XV, which may contribute to dorsal closure (Liu et al. 2008). I therefore also tested DEcadΔβ::αCatΔVH1, a construct generated by Ritu Sarpal in our lab which removes only 21 amino acids of the Armadillo binding site of DE-cadherin as compared to the 61 amino acid
deletion in DEcadΔ61::αCat. DEcadΔβ::αCatΔVH1 also has a deletion of the VH1 region of α-Catenin which binds to Armadillo and can facilitate α-Catenin dimerization (Pokutta and Weis 2000). This construct should therefore specifically prevent Armadillo recruitment to the adherens junctions and also prevent the dimerization of α-Catenin. In addition, DEcadΔβ::αCatΔVH1 as well as other constructs (except DEcadΔ61::αCat) were expressed out of the same genomic insertion site (attP2) to facilitate comparable expression levels. DEcadΔ61::αCat was inserted randomly into the third chromosome (Pacquelet and Rørth 2005), and hence its expression level may vary from the other constructs.

Figure 6. Schematic of UAS-transgenes used in this study. Diagram shows full-length DEcad, and full length DEcad fused to full length αCat. The DEcadΔ61::αCat fusion protein has a 61 aa deletion at the C-terminus of the DEcad cytoplasmic tail that covers the Armadillo binding site and a Myosin XV interaction sequence. DEcadΔβ::αCatΔVH1 lacks 21 amino acids of the Armadillo binding site of DEcad and the Armadillo-binding VH1 region of α-Cat. All constructs have been inserted at the attP2 site except for DEcadΔ61::αCat, which was inserted randomly into the third chromosome (Pacquelet and Rørth 2005).

I first tested whether the chimeric proteins localized to the plasma membrane and recruited Armadillo to adherens junctions. I expressed the constructs in epidermal stripes using en-Gal4 in a shg^{R69} mutant background. I found that all constructs were expressed and localized to the adherens junctions (Figure 6). Both DEcad and DEcad::αCat recruited Armadillo as expected. In
contrast, both fusion proteins that lack the Armadillo binding site in DE-cadherin did not localize Armadillo at the membrane and instead Armadillo was found in the cytoplasm. DEcadΔ61::αCat did not localize Armadillo to the membrane even though the VH1 domain, which is responsible for Armadillo binding within α-Catenin is still present in DEcadΔ61::αCat. Previous studies found that the VH1 region of α-Catenin can dimerize and bind to β-catenin/Armadillo in a mutually exclusive fashion (Pokutta and Weis 2000). My findings therefore suggest that the VH1 region of α-Catenin in DEcadΔ61::αCat is preferentially dimerized, which would exclude Armadillo binding and recruitment to adherens junctions. A recent study also confirms the preferential dimerization of the VH1 domain of α-Catenin (Bianchini et al. 2015). Taken together, all constructs localized to adherens junctions and the fusion proteins lacking the Armadillo binding site did not recruit Armadillo to the membrane.
Figure 7. Expression of DEcad and DEcad-αCat chimeras in epidermal stripes in shg<sup>R69</sup> mutant background. Panels show stage 15 embryos stained for DEcad (red), Arm (green), Merge (Yellow). (A-B) DEcad and DEcad::αCat both localizes Armadillo to the membrane. (C-D) In contrast, both constructs lacking the Armadillo binding site fail to recruit Armadillo to the membrane.
Next, I tested whether overexpressing the UAS transgenes caused lethality (Figure 7). Lethality counts from ubiquitous overexpression of chimeras using da-Gal4 revealed that both DEcad and DEcad::αCat when overexpressed are pupal lethal. Since both constructs can bind Armadillo, it is likely that they titrate Armadillo to the membrane, preventing it from acting as an effector in Wg signalling and therefore causing lethality as has been shown previously (Sanson et al. 1996). Wild-type control and DEcadΔβ::αCatΔVH1 were not lethal. Interestingly, however, overexpression of DEcadΔ61::αCat led to embryonic lethality. Cuticle preparations from embryos overexpressing DEcadΔ61::αCat showed 43% embryos with head defects, 4% with head and dorsal defects, 1% with head and ventral defects, and 52% showed head defects in conjunction with dorsal and ventral defects (Figure 8). Why this construct, although not titrating Armadillo, causes embryonic defects is not known. Because of the observed overexpression defects of DEcadΔ61::αCat, including defects in dorsal closure and the ventral epidermis I would not expect to see a complete rescue when DEcadΔ61::αCat is expressed in the shotgun or armadillo mutants. On the other hand, all construct except for DEcadΔ61::αCat, could completely rescue embryonic defects of shotgun and armadillo mutants since they are not embryonically lethal when overexpressed.
Figure 8. Lethality counts of embryos overexpressing transgenes. UAS-transgenes were over-expressed with daGal4 and followed through development. Overexpression of DEcad and DEcad::αCat showed predominantly pupal lethality. DEcadΔ61::αCat is embryonically lethal, whereas DEcadΔβ::αCatΔVH1 is not lethal when overexpressed.

Figure 9. Cuticle preparations of embryos overexpressing DEcadΔ61::αCat. Embryos overexpressing DEcadΔ61::αCat with daGal4 were embryonically lethal. (A) Wildtype embryo. (B) Embryo overexpressing DEcadΔ61::αCat with severe head defects (arrow). (C) Embryo lacking dorsal and ventral cuticle (right image).
Immunoblot of transgenic constructs expressed with da-Gal4 reveal that DE-cadherin and all fusion proteins accumulated in the embryo when overexpressed (Figure 9). However, DEcadΔβ::αCatΔVH1 and DEcad::αCat appeared to accumulate at a higher level than DEcadΔ61::αCat. Since DEcadΔ61::αCat has been randomly inserted into the third chromosome, it might explain why it does not accumulate to the same level as transgenes that have been inserted into the attP2 site. Posttranslational modification of DE-cadherin in which the extracellular region is cleaved at its cysteine-rich region produces a N-terminal fragment of ~150kDa and a C-terminal fragment of ~80kDa (Oda and Tsukita 1999). A strong band for endogenous DEcad is seen in all embryos overexpressing transgenes.

Figure 10. Immunoblot showing the overexpression of DEcad and DEcad-αCat fusion proteins. DEcad and DEcad α-Cat fusion proteins were expressed under the control of da-Gal4 and detected by immunoblotting 60μg of embryonic lysates with antibodies to α-Catenin (upper panel), DEcad (middle panel). β-tubulin serves as loading control. All expressed protein show substantial levels of overexpression. All bands ran lower than the ladder.
In conclusion, all UAS-transgenes were expressed and accumulated in the embryo. The embryonic lethality and defects observed when DEcadΔ61::αCat were overexpressed might interfere with the ability of this construct to rescue embryonic defects in *shotgun* and *armadillo* mutants. Also, all constructs localize to adherens junctions and the fusion proteins lacking the Armadillo binding site did not recruit Armadillo to the membrane.
Part 2- Armadillo recruitment to adherens junctions is dispensable in the ventral epidermis but not dorsal closure

The first set of rescue experiments were conducted in a $shg^{R69}$ zygotic mutant background. Maternal/zygotic shotgun mutants result in a loss of epithelial integrity throughout the embryo early in development. In contrast, zygotic shotgun mutants have epithelial defects but many epithelial cells develop comparatively normally until later stages due to the presence of maternal DEcad (Tepass et al. 1996). Embryos of the null allele $shg^{R69}$ are embryonic lethal, and show severe head defects, a consistent loss of ventral cuticle, and a failure of dorsal closure (Figure 10). Since dorsal closure and the formation of the ventral epidermis are both disrupted when adherens junctions are compromised, it was these processes that were rescue by using the DEcad-αCat fusion proteins listed in Table 2.

DEcad, DEcad::αCat, DEcadΔ61::αCat, and DEcadΔβ::αCatΔVH1 were expressed in $shg^{R69}$ mutant embryos with the mat67Gal4, a maternal driver, daGal4, a ubiquitous driver, or enGal4, a segmental driver. No driver was used as control. The cuticle preparations from these rescue crosses were assessed by scoring for dorsal and ventral defects. Ventral defects were scored based on the number of intact abdominal denticle belts, where an intact ventral epidermis was given a score of 0 and a completely disrupted ventral cuticle was given a score of 8. Dorsal holes were given scores based on whether there was an intact dorsal cuticle (no defect - 0), no hole but a defect was apparent in the cuticle at the dorsal midline indicative of a defect in dorsal closure (minor defect-1), a dorsal hole that is contained within two segments or less (small hole-2), a hole that extends beyond two segments but is not extending across the entire embryo (partial
hole-3), and, finally, a hole that starts from the head and ends at the spiracles so that the embryo is completely open on the dorsal side (complete hole-4) (Figure 11).

Figure 11. Cuticle preparations and staining of zygotic shgR69 mutant embryos. (A) Cuticle preparation of shgR69 mutant embryos show lack of ventral and dorsal cuticle with only the lateral cuticle remaining. (B) shgR69 mutant embryos stained for Dlg (red), DEcad (blue) and Ed (green) show strongly reduced DEcad levels and a lack of ventral epidermis and a failure in dorsal closure.
Figure 12: Examples of cuticle defects and scoring of defects. Embryonic cuticles of experimental embryos were assessed by scoring separately for dorsal and ventral defects. Ventral defects were scored based on the number of intact denticle belts (0-8). Dorsal holes were given scores based on whether there was an intact cuticle (no defect - 0), a defect in the dorsal cuticle without an obvious hole (minor defect - 1), a dorsal hole retained within one or two segments (small hole - 2), a hole that extends beyond two segments (partial hole - 3), and a hole that starts from the head and ends at the spiracles, that is an embryos that is complete open dorsally (complete hole - 4).
The first rescue experiment I conducted was a negative control: the expression of UAS-transgenes in $shg^{R69}$ mutant background without a driver (Figure 12). I observed a moderate rescue of dorsal and ventral defects in this no driver control suggesting that UAS transgenes have leaky expression even in the absence of a Gal4 driver. I found that DEcad and DEcad::αCat rescue ventral defects better than fusion proteins lacking the Armadillo binding site. Together, this suggests that all transgenes have leaky expression which is sufficient to elicit a partial rescue of $shg^{R69}$ mutants.
Figure 13. Rescue of $shg^{R69}$ mutants with DEcad-αCat fusion proteins UAS-transgenes were expressed in $shg^{R69}$ mutant background with the indicated Gal4 drivers and assessed by scoring for dorsal and ventral defects using embryonic cuticle preparations. (A) $shg^{R69}$ mutant embryos containing UAS-transgene and no Gal4 driver. All constructs apparently showed leaky expression in the absence of a Gal4 driver and significantly rescued dorsal and ventral defects of $shg^{R69}$ embryos. DEcad and DEcad::αCat rescue ventral and dorsal defects better than fusion proteins lacking the Armadillo binding site. (B,C) Expression of transgenes with enGal4 or daGal4 show levels of rescue of the $shg^{R69}$ mutant defects similar to the no driver control (A), suggesting that these two drivers do not lead to effective expression of UAS-transgenes including DEcad. DEcad and DEcad::αCat showed a similar strong rescue of ventral defects than
fusion proteins lacking the Armadillo binding site. (D) The expression of UAS-transgenes was driven by mat67-Gal4 and da-Gal4 in the shg^{R69} mutant background. All transgenes significantly rescued the dorsal and ventral defects in shg^{R69} mutants. DEcadΔβ::αCatΔVH1 rescued most dorsal defects but its rescue activity was weaker than DEcad and DEcad::αCat. In the ventral epidermis a near complete rescue was observed with all constructs. Data represent mean ±SD. The asterisks indicate significant difference from the mean of shg^{R69} mutants using unpaired t-test (p< 0.0001).

Next, I used en-Gal4, the patterned driver used by Gorfinkiel and Arias (2007) to drive the expression of the UAS-transgenes in epidermal stripes in shg^{R69} mutant background (Figure 12) as a replicate of their experiment. I found no major difference to the no driver control, even though en-Gal4 should have two components of expression, one is the leaky expression of the UAS-transgenes and the second is the en-Gal4 driven component in segmental stripes. In fact, whereas DEcad and DEcad::αCat showed a significant rescue of dorsal defects in shg^{R69} mutants, the difference between en-Gal4 shotgun embryos and en-Gal4 shgmut embryos expressing the fusion proteins lacking the Armadillo-binding site was not significant in this case and in contrast to the no driver control. These differences in statistical significance may be due to some variation in the phenotypic severity of the shotgun mutant phenotype between experiments. These results are similar to the findings of Gorfinkiel and Arias (2007) in that constructs that fail to recruit Armadillo show less rescue than DEcad or DEcad::αCat. However, my observations indicated that the majority of the rescue observed by these authors arose from leaky expression rather than the use of the enGal4 driver. Therefore, expression of the UAS-transgenes in epidermal stripes in shg^{R69} mutant background can partially rescue dorsal and ventral defects of shotgun mutants.

The second driver I employed was daGal4 to test whether ubiquitous high level expression rather than the expression in epidermal stripes of the UAS-transgenes leads to a more substantial rescue
of dorsal closure and the ventral epidermal defects in shotgun mutants (Figure 12). Surprisingly, dorsal closure and ventral defects of shgR69 mutants could not be rescued more extensively by expressing DEcad or any of the chimeric proteins than what was seen in the no driver control. Only marginal improvements were observed for DEcadΔβ::αCatΔVH1 in dorsal closure (Figure 12). The fact that DEcad expression with daGal4 did not rescue shotgun mutants better than the no driver control was particularly puzzling as the expression of DEcad under the control of the ubiquitin promoter fully compensates for the loss of shotgun function (Oda and Tsukita 2001). da-Gal4 is a zygotic driver that activates UAS transgenes during gastrulation after which expression increases over time. It is possible therefore that da-Gal4 does not express transgenes early enough at sufficiently high levels to elicit a better rescue. I therefore combined daGal4 with a maternal driver in my next set of experiments.

Next I drove the expression of UAS-transgenes in shotgun mutants with da-Gal4 and the maternal driver mat67-Gal4 to ensure that UAS-transgenes are activated in early embryos (Figure 12 & 13). This expression strategy led to a nearly complete rescue of dorsal and ventral defects in shotgun mutant embryos expressing DEcad and DEcad::α-Cat. Also DEcadΔβ::αCatΔVH1 almost fully rescued ventral defects of shotgun mutants whereas dorsal closure defects where rescued only partially, but substantially more so than in the no driver control. DEcadΔ61::αCat was not quantified in this experiment since overexpression of this construct with da-Gal4 has a phenotype of its own and would interfere with the ability of this construct to cause a rescue of the shotgun mutant phenotype. Interestingly, however, DEcadΔβ::αCatΔVH1 showed a strong rescue of ventral defects suggesting that Armadillo acts primarily as a linker between DE-cadherin and α-Catenin in the maintenance of the ventral epidermis. In contrast, since DEcadΔβ::αCatΔVH1 could not fully rescue dorsal closure defects
“f shotgun, it would seem that Armadillo must play a role at adherens junctions necessary for dorsal closure that is different of its function as a physical linker between DE-cadherin and α-Catenin.

**Figure 14. Rescue of shg<sup>R69</sup> mutants with ubiquitous expression of UAS-transgenes.** Cuticle preparations showing: (A) wildtype cuticle, (B) shg<sup>R69</sup> mutants showing complete lack of dorsal and ventral cuticle with only the lateral cuticle remaining, (C) Rescued embryo overexpressing DEcadΔβ::αCatΔVH1 with mat67-Gal4 and da-Gal4 in shg<sup>R69</sup> mutant background showing complete rescue of ventral and dorsal cuticle except retaining head defects.

**Part 3 – Armadillo contributes to dorsal closure in a role that is independent from its function as a linker in the cadherin-catenin complex.**

To more directly address the question whether Armadillo acts simply as a linker between DE-cadherin and α-Catenin or has other roles in dorsal closure, I carried out a set of rescue experiments in which the UAS transgenes were expressed in arm<sup>d</sup> mutants. arm<sup>d</sup> mutant embryos show ventral defects that result from a loss of Wingless signalling (a lawn of denticles) and also fail to undergo dorsal closure (Figure 14). If fusion proteins that prevent Armadillo recruitment to the membrane can rescue dorsal closure defects in the absence of Armadillo, it would suggest that Armadillo functions primarily as a linker between DE-cadherin to α-Catenin. A failure of
rescue, on the other hand, would indicate that Armadillo has other functions in dorsal closure, either at adherens junctions or independent of adherens junctions. Since Wingless signalling is also required for dorsal closure (Orsulic and Peifer 1996; McEwen et al. 2000; McEwen and Peifer 2000; Morel and Arias 2004), it could be challenging to separate the role of Armadillo in adhesion from its role in Wnt signalling in dorsal closure.

I rescued arm\(^4\) zygotic mutants without a driver, with en-Gal4 and with da-Gal4 (Figure 14). When UAS-transgenes are expressed in arm\(^4\) mutants, they retain the Wnt phenotype in the ventral epidermis, showing a lawn of denticles. In contrast they show variability in the rescue of dorsal closure. Degree of rescue in these experiments was scored as a ratio of the length of dorsal cuticle that remains, over the length of the embryo from the spiracles to the head. DEcad\(\Delta 61::\alpha\)Cat was not examined in these rescue experiments since it already has a defect when overexpressed and would be very difficult to assess.

Interestingly, I found that DEcad::\(\alpha\)-Cat can rescue dorsal defects of arm\(^4\) to some degree when expressed ubiquitously under the control of da-Gal4, suggesting that Armadillo functions as both a linker in the cadherin-catenin complex and in Wingless signalling in dorsal closure. Furthermore, DEcad\(\Delta \beta::\alpha\)Cat\(\Delta VH1\) rescued dorsal closure defects of armadillo mutants more so than DEcad::\(\alpha\)-Cat. The better rescue of DEcad\(\Delta \beta::\alpha\)Cat\(\Delta VH1\) could be rationalized by assuming that as DEcad\(\Delta \beta::\alpha\)Cat\(\Delta VH1\) does not bind Armadillo and therefore cannot titrate it away from its function in Wingless signalling. DEcad\(\Delta \beta::\alpha\)Cat\(\Delta VH1\) would restore adhesion like DEcad::\(\alpha\)-Cat but would allow residual maternal Armadillo to be fully available for Wnt signalling leading to a stronger rescue of dorsal closure. Armadillo recruitment to the membrane is relevant and interferes with Wnt signalling in this context which is suggested by my finding
that expression of DE-cadherin under the control of daGal4 enhances the dorsal closure defects of *armadillo* mutants. Based on these results, I do not think that one could infer a role for Armadillo in addition to linker and in Wnt signalling in this setup.
Figure 15. Examples of cuticles and quantification of the rescue of arm\textsuperscript{d} zygotic mutant embryo by expressing UAS-transgenes. (A) \textit{arm}\textsuperscript{d} zygotic mutant embryo displays a lawn of denticles on the ventral side and a complete dorsal holes. Embryo with ubiquitous expression of DEcad\textsuperscript{Δβ::αCatΔVH1} shows partial rescues of dorsal closure phenotype. (B) UAS-transgenes were expressed with da-Gal4, en-Gal4, and no driver in \textit{arm}\textsuperscript{d} mutant embryos, and assessed by scoring for dorsal defects using embryonic cuticle preparations. A small but significant rescue (P<0.0001) of dorsal closure was observed in the no driver control and with en-Gal4 in \textit{armadillo} mutant embryos expressing DEcad\textsuperscript{Δβ::αCatΔVH1}, when constructs were expressed with da-Gal4, significant rescues were seen with DEcad::αCat and DEcad\textsuperscript{Δβ::αCatΔVH1}, with the DEcad\textsuperscript{Δβ::αCatΔVH1} rescue being significantly stronger than the rescue seen with DEcad::αCat (P<0.0001).
Chapter 4
Discussion and Future Directions

Whether Armadillo/β-catenin’s primary function is to link DE-cadherin to α-Catenin or it has further roles in regulating adherens junction assembly and stability remains a controversial issue. Current literature would suggest that β-catenin either functions only as a linker or has also a regulatory role in adherens junctions stability and that the latter is highly tissue dependent (Sarpal et al. 2012; Pacquelet and Rørth 2005; Gorfinkiel and Arias 2007; Tamada et al. 2012; Langevin et al. 2005; Mirkovic et al. 2011; Speder et al. 2006). To further investigate the function of Armadillo in morphogenesis, I have studied its role in dorsal closure and its function in the development of the ventral epidermis. The question I tested was whether Armadillo is required at adherens junctions during dorsal closure and the formation of the ventral epidermis in *Drosophila*, and whether this requirement goes beyond physically connecting DE-cadherin to α-Catenin. We found that Armadillo functions primarily as a linker between DE-cadherin and α-Catenin during the formation of the ventral epidermis. However, Armadillo appears to be required at adherens junctions for dorsal closure apart from linking DE-cadherin to α-Catenin. This second role of Armadillo may not be fully explained through the contribution Armadillo makes to Wnt signalling, which is also required for normal dorsal closure (Morel and Arias 2004).

Ventral Epidermis

Ventral defects of *shotgun* mutants can be rescued by fusion proteins of DE-cadherin and α-Catenin lacking an Armadillo binding site, suggesting that in the ventral epidermis, β-catenin
functions primarily as a linker between DE-cadherin and α-Catenin and any regulatory role of Armadillo at adherens junctions may not be essential for this tissue. These results are surprising as the ventral epidermis is a highly dynamic tissue that undergoes many morphogenetic processes that require adherens junction function and remodelling including germ band extension, cell division and neuroblast ingestion. Maintaining epithelial integrity while these processes go on is highly dependent on adherens junction function (e.g. Tepass et al. 1996). Armadillo is also known to function primarily as a physical linker in other tissues, like imaginal disc epithelia or in the Drosophila ovary. Integrity of the follicular epithelium, collective migration of the border cells, and oocyte positioning defects of armadillo and α-Catenin mutants have been fully rescued by the expression of a DEcad::αCat fusion protein, suggesting that the primary function of Armadillo is to link DE-cadherin and α-Catenin (Sarpal et al. 2012; Pacquelet and Rørth 2005). In contrast to the follicular epithelium, which is a rather a static tissue, border cell migration and oocyte positioning are both dynamic processes that require remodeling of adherens junctions. Since in this context Armadillo is not required for adherens junction function, it suggests that other components of the adherens junction, such as α-Catenin, could be the target for regulatory mechanisms that facilitate adherens junction dynamics during these morphogenetic processes (Sarpal et al. 2012; Pacquelet and Rørth 2005). To identify which domains of α-Catenin or which α-Catenin interaction partners other than Armadillo might be necessary to regulate adherens junction function in the ventral epidermis, further studies could focus on Drosophila embryos imaged live to carefully examine adherens junction-dependent events such as mesoderm invagination or germband extension (Oda and Tsukita 2001; Blankenship et al. 2006; Tamada et al. 2012). For example, siRNA directed against 5’UTR of α-Catenin can be injected into early embryos to knock down α-Catenin expressing endoDEcad::GFP. Preliminary work from our lab has shown that these embryos exhibit a loss of
adherens junctions and breakdown of the ectoderm. Full length α-Catenin, expressed from a construct lacking the 5'UTR, can restore adherens junctions and tissue integrity of knockdown embryos. The pilot experiment in which DEcadΔβ::αCatΔVH1 was used to rescue α-Catenin mutants also restored adherens junctions and tissue structure suggesting again that Armadillo is primarily behaving as a linker between DE-cadherin and α-Catenin in respect to maintaining epithelial tissue structure in early embryos. However, germband extension and mesoderm invagination and other morphogenetic processes have not yet been carefully analyzed in such embryos. In particular, Tamada and colleagues have proposed that Abelson kinase phosphorylates Armadillo at Y667 is important for Armadillo turnover, which promotes cell rearrangement that drives axis elongation. Thus, it will be interesting to carefully examine the process of germband extension in Armadillo (or DE-cadherin or α-Catenin) compromised embryos that express DEcadΔβ::αCatΔVH1.

Maternal expression of DE-cadherin in conjunction with ubiquitous expression was needed to elicit a rescue of shotgun mutants. Similar level of rescue was observed with the expression of DE-cadherin without a driver, with en-Gal4 and da-Gal4. This suggests that leaky expression could be responsible for much or the entire rescue observed with enGal4 and daGal4. The observation that maternal expression of DE-cadherin rescues much better suggest that an earlier expression is required to elicit a rescue. This is because morphogenetic movements in the ventral epidermis occur prior to dorsal closure. The ubiquitous expression with da-Gal4 may not have been expressing the transgenes at earlier stages when morphogenetic movements challenge the integrity to the ventral epithelium. In future experiments it would be interesting to identify when during development da-Gal4 driven DE-cadherin constructs show protein levels above
background to identify a time point before which significant expression has to occur to ensure strong rescue of the zygotic shotgun mutant defects in the ventral epidermis.

Rescuing shotgun mutants with UAS-trangenes using no driver (as well as en-Gal4 or da-Gal4) showed a pattern of rescue of ventral defects where DEcad and DEcad::αCat were more effective than the fusion proteins lacking the Armadillo binding site. In contrast mat67-Gal4 driven constructs showed similar rescue level for DEcad and DEcad::αCat as well as DEcadΔβ::αCatΔVH1. These findings suggest that leaky expression leads to threshold levels of protein expression that provides a partial rescue and that under those conditions differences between constructs that bind Armadillo and those that don’t may become more apparent. This could point to roles for Armadillo at adherens junctions that are redundant under normal expression conditions. One possibility is a compromised ability of constructs that fail to bind to Armadillo to be transported normally to adherens junctions. β-catenin is known to bind to newly synthesized E-cadherin in the endoplasmic reticulum and be co-transported to the plasma membrane (Chen et al. 1999). Work in the Drosophila notum suggested that Armadillo’s interaction with the exocyst complex is important for normal DE-cadherin delivery to the adherens junction (Langevin et al. 2005). Moreover, β-catenin binding to E-cadherin shields a PEST sequence motif preventing its proteasomal degradation (Hinck et al. 1994). These findings are consistent with the conclusion that higher levels of DEcadΔβ::αCatΔVH1 expression are required to compensate for the failure to interact with Armadillo.
Dorsal Closure

In embryos deficient for DE-cadherin and Armadillo, dorsal closure was only partially rescued by fusion proteins that directly link DE-cadherin to α-Catenin and lack the β-catenin binding sites. These results suggest that apart from being a linker between DE-cadherin and α-Catenin, Armadillo is necessary at adherens junctions for dorsal closure. However, the inability to see a complete rescue in armadillo mutants may be due to compromising Armadillo function in Wingless signalling (Orsulic and Peifer 1996). Armadillo is a bifunctional protein, it is an integral component of adherens junctions and at the same time a key nuclear effector of canonical Wingless signalling (Huber et al. 1997; Nusse 1997; Clevers and Nusse 2012; Valenta et al. 2012). There are two pools of Armadillo/β-catenin: in the absence of Wingless signalling, Armadillo is only found at adherens junctions and any cytoplasmic Armadillo is degraded. However, in the presence of Wingless signalling, Armadillo is no longer targeted for degradation, it accumulates in the cytoplasm and translocates to the nucleus where it helps express Wingless target genes (Huber et al. 1997; Nusse 1997; Clevers and Nusse 2012; Valenta et al. 2012). In zygotic null mutants of Armadillo, there is residual Armadillo provided by the maternal contribution. This residual Armadillo can presumably function in Wingless signalling or at adherens junctions but not in both since it has overlapping binding sites for DE-cadherin and Wingless effectors that ensures Armadillo only functions as part of one complex (Orsulic and Peifer 1996). If Armadillo is present at low levels it predominantly or exclusively appears to act in adhesion because zygotic armadillo mutant embryos display a strong loss of Wingless signalling phenotype but largely retain epithelial integrity, which depends on an intact cadherin-catenin complex. Armadillo-dependent Wingless signalling is required for the polarisation of the dorsal-most epidermal cells in the plane of the epithelium as an important aspect of dorsal closure to occur properly (Morel and Arias, 2004). Therefore, the dorsal closure defects may be
due to the lack of Armadillo necessary for Wingless signalling rather than at adherens junctions. Why β-catenin has evolved to incorporate two distinct functions remains an open question. Perhaps since β-catenin can only be part of one complex and is preferentially recruited to adherens junctions, it can define an activation threshold for Wingless signalling in the presence of adherens junctions.

The inability of fusion proteins that directly link DE-cadherin to α-Catenin but lack the β-catenin binding site to rescue dorsal closure suggests that Armadillo at adherens junctions has a role in addition to its function as a linker that is required for proper dorsal closure. This is consistent with the study by Gorfinkiel and Arias (2007) that also found that Armadillo plays a separate and essential role other than its role in linking DE-cadherin to α-Catenin at adherens junctions during dorsal closure. They showed that embryos mutant for a dominant negative allele of shotgun (shg$^{g317}$) exhibit dorsal holes that can be rescued by the expression of DEcad::αCat driven in epidermal stripes or in the amnioserosa. However, a fusion protein lacking the β-catenin binding site, DEcadΔ61::αCat could not rescue the dorsal closure defects in shotgun mutant embryos. Additionally, DEcad::αCat does not rescue dorsal closure defects in armadillo zygotic null mutant embryos when expressed either ubiquitously or in stripes. The authors concluded that the lack of Armadillo at adherens junctions prevented normal dorsal closure suggesting that Armadillo has a role in dorsal closure that is separate from its role as a linker between DE-cadherin to α-Catenin (Gorfinkiel and Arias 2007). One difficulty of this study was that it did not test whether ubiquitous expression of DEcadΔ61::αCat or leaky expression of the UAS construct in the absence of a Gal4 driver can rescue dorsal closure in shotgun mutants, or whether overexpression causes a phenotype. I found that when overexpressed, this construct leads to embryonic death, with embryos showing severe head, dorsal and ventral defects. Therefore, for
my study, I also used DEcadΔβ::αCatΔVH1, which removes only 21 amino acids of the Armadillo binding site of DE-cadherin as compared to the 61 amino acid deletion in DEcadΔ61::αCat. DEcadΔβ::αCatΔVH1 also has a deletion in the Armadillo-binding VH1 region of α-Catenin preventing α-Catenin binding to Armadillo and homo-dimerization (Pokutta and Weis 2000). This construct does not cause lethality when overexpressed, proving to be a better fusion protein to use to rescue shotgun mutants. One reason why DEcadΔ61::αCat may lead to embryonic lethality may be due to its large deletion which compromises its interaction with other proteins. One example is the Drosophila Myosin XV homolog, Sisyphus, which is known to bind in the region of DE-cadherin that has been deleted in DEcadΔ61::αCat. DE-cadherin binds to the Sisyphus FERM domain and is transported to filopodia at the leading edge where it is important to form transient cell-cell contacts and resolve them to form more permanent contacts as dorsal closure proceeds (Liu et al. 2008). One further concern is that Gorfinkel and Arias (2007) conducted their experiments in a dominant negative allele of shotgun rather than a null allele, which could lead to different results. The authors also did not include a no-driver control so that it is unclear to what extent leaky expression contributed to the observed rescue activity of different constructs. I found that all the constructs have leaky expression, and this expression is enough to give a partial rescue of dorsal and ventral defects. Nevertheless, their conclusions are consistent with my results; e.i. that Armadillo seems to be necessary at adherens junctions for dorsal closure aside from linking DE-cadherin to α-Catenin.

Several studies have proposed that Armadillo at adherens junctions is important for the regulation of adherens junction stability in addition to its essential role in linking DE-cadherin to α-Catenin. These studies focused on Armadillo’s function in trafficking adherens junctions components (Chen et al. 1999; Langevin et al. 2005), establishing left-right asymmetry and
ommaticidial rotation (Speder et al. 2006; Mirkovic et al. 2011), as well as Armadillo phosphorylation that leads to dissociation of the cadherin-catenin complex (Ozawa and Kemier 1998; Huber and Weis 2001; Piedra et al. 2003; Brembeck et al. 2004; Tominaga et al. 2008; Tamada et al. 2012).

β-catenin is known to contribute to cadherin delivery to the plasma membrane. In MDCK cells, it was found that E-cadherin mutants that are unable to bind β-catenin, are inefficiently delivered to the plasma membrane (Chen et al., 1999), and accumulate in post-Golgi compartments and early endosomes before being delivered to lysosomes due to a sorting mechanism that is dependent upon a dileucine motif in the cadherin juxtamembrane domain (Miyashita and Ozawa 2007). Moreover, in the cells of the notum of the Drosophila wing imaginal disc, Armadillo was shown to provide a landmark for the delivery of DE-cadherin from the recycling endosomes to the adherens junctions by interacting with the exocyst complex (Langevin et al., 2005). Therefore, there is evidence in both vertebrate and invertebrates systems of β-catenin participating in trafficking cadherin to adherens junctions.

β-catenin also functions in promoting ommatidial rotation and establishing left-right asymmetry. Nemo kinase-dependent serine-threonine phosphorylation of Armadillo is important for promoting ommatidial rotation (Mirkovic et al. 2011). Also, unconventional type ID myosin (MyoID) physically interacting with Armadillo and acts as a left-right determinant responsible for the clockwise rotation of genitalia in Drosophila males (Speder et al., 2006). Therefore, β-catenin has additional roles at adherens junctions that are necessary for Drosophila development.
Apart from trafficking adherens junction components and promoting ommatidial and genital rotation, phosphorylation of β-catenin by various kinases regulate structural integrity of cadherin-based junctions often causing weakening of the β-catenin interaction with cadherin or α-Catenin and directing β-catenin towards signalling. Phosphorylation of β-catenin on tyrosine 142 by Fyn, Fer and cMet kinases reduce its affinity for α-Catenin in vitro, thereby impairing β-catenin’s adhesive function and shifting β-catenin’s function towards signalling (Ozawa and Kemier 1998; Piedra et al. 2003; Brembeck et al. 2004; Tominaga et al. 2008). Also, another well-studied β-catenin phosphorylation site is tyrosine 654 in the last Armadillo repeat of β-catenin. In mammalian cell lines, it has been found that phosphorylation of β-catenin at tyrosine 654 reduces its binding to cadherin and enhances its function in signalling by stimulating the association of β-catenin with the basal transcription factor TATA-binding protein (Piedra et al. 2001). The negative charge of a phosphorylated tyrosine 654 interferes with the key aspartate residues in cadherin, reducing the cadherin-β-catenin interaction (Huber and Weis 2001). In vivo, mouse mutants expressing a phospho-mimetic form of β-catenin (Y654E) showed aberrant activation of Wnt signalling resulting in embryonic death (van Veelan et al., 2011). However, since phospho-mimetic mutants do not affect the structure of adherens junctions, it suggests that there are compensatory mechanisms that exists in vivo (van Veelan et al. 2011). *C. elegans* express four distinct β-catenin paralogs, out of which only HMP-2 functions in cell adhesion (Korswagen et al. 2000). HMP-2 tyrosine 599 is homologous to human and mouse β-catenin tyrosine 654. Phosphorylation of HMP-2 at tyrosine 599 may reduce its interaction with region II of the HMR-1 cadherin (Choi et al. 2015).

In *Drosophila*, tyrosine 667 in Armadillo is homologous to tyrosine 654 in mammalian β-catenin. Phosphorylation of Y667 is not required during oogenesis since phosphomutant
Armadillo Y667F can rescue epithelial integrity in all cadherin-mediated processes in the *Drosophila* ovary (Pacquelet and Rorth 2005). Also, since DEcadΔ61::αCat can rescue *shotgun* and *armadillo* mutant defects in the ovary, regulation of adherens junctions must not be occurring through Armadillo phosphorylation at this site (Pacquelet and Rorth 2005). However, in other processes, such as germ band extension, phosphorylation of Armadillo at Y667 by Abelson tyrosine kinase is important for Armadillo turnover, which promotes cell rearrangement that drives axis elongation (Tamada *et al.* 2012). Therefore, tyrosine phosphorylation of β-catenin/Armadillo is well known to impact adherens junction remodeling.

In processes as dynamic as dorsal closure, where cell-cell contacts need to be resolved and reformed, Armadillo phosphorylation may be required to regulate adherens junctions. Since there is partial rescue of constructs that prevent recruitment of Armadillo to the membrane and not a complete rescue, regulation of Armadillo may work in parallel with another components of adherens junctions that are required for adherens junction remodelling. It could also be that the UAS-transgenes need to be expressed earlier in development to see a complete rescue. This would be similar to the results from *shotgun* mutants, when a complete rescue was only observed when the UAS-transgenes were expressed early during development. For these experiments, the UAS-transgenes can be expressed with a maternal driver. If DEcadΔβ::αCatΔVH1 is again unable to completely rescue *armadillo* mutants it would suggest that Armadillo plays a key role at adherens junctions that is necessary for dorsal closure and that is independent of its function as a physical linker between DE-cadherin and α-Catenin.

β-catenin is a complex molecule that is involved in cell adhesion and is also a key nuclear effector of Wnt signalling. β-catenin is also known to have several roles in cell-cell adhesion
aside from simply linking DE-cadherin to α-Catenin. However, in some processes its only essential function is as a physical linker between DE-cadherin and α-Catenin. In such cases, other components of adherens junctions are needed to regulate cell-cell adhesion. Whether β-catenin functions in regulating adherens junctions or behaves only as a linker is highly tissue specific. In this thesis I further investigated the function of Armadillo in morphogenesis by examining its role in dorsal closure and in the development of the ventral epidermis. I found that Armadillo functions primarily as a linker between DE-cadherin and α-Catenin during the formation of the ventral epidermis. In contrast, Armadillo is necessary at adherens junctions for dorsal closure apart from linking DE-cadherin to α-Catenin.
References


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