Tension at the leading edge: differential expression of the cell adhesion molecule Echinoid controls epithelial morphogenesis in *Drosophila*

Caroline Laplante
Department of Biology
McGill University
Montréal, Québec, Canada

Doctor of Philosophy

April 2008

A thesis submitted to McGill University in partial fulfillment of the requirement of the degree of Doctor of Philosophy

Acknowledgements

I wish to express my sincere gratitude to the people who have shown outstanding support at different times and in varied ways throughout of the course of this degree. To Professor Laura Nilson, for respecting my research interests and allowing the free expression of my creativity in the design and execution of my project. Through Laura's genuine support, I have developed valuable scientific and organizational skills and most importantly, a good sense of diplomacy. To Professor Paul Lasko, for his scientific and career advice. I am grateful to the members of the Nilson, Lasko and Schöck labs and all others who brought constructive criticisms to my work. To Phoenix Bouchard Kerr, our lab technician, for her valuable support in ensuring the smooth running of essential lab operations. To Beili Hu for injection of transgene and Mr. Lee for making the fly food. To the members of my curriculum committee: François Fagotto, Frieder Schöck and Jackie Vogel for their feedback. To my boyfriend John Grey who has taught me to embrace physics and incorporate optics into my biology work. To my best friends (in alphabetical order) Jean-François Boisclair Lachance, Jan-Michael Kugler, Jocelyn Moore, Gritta Tettweiler and Travis Thomson for every minute shared in their company.

I am grateful to my parents, Murielle and Léo, and my brother David for their constant encouragements, keen interest and for understanding that studying fruit flies is an acceptable career choice. I would like to thank my hockey team, the Knockout, for their exemplary courage and constant improvements throughout the years have been a great source of inspiration and motivation.

This work was initiated during my undergraduate studies with the financial support of an NSERC undergraduate award and most recently by a generous scholarship from the National Cancer Institute of Canada. The McGill University Alma Howard Alumni and department of Biology have provided travel awards for the presentation of my work at international conferences and thus contributed greatly to my learning experience. This research would not have been possible without reagents provided by the laboratories of Celeste Berg, Richard Fehon, David Hipfner, Daniel Kiehart, Daisuke Yamamoto and Jennifer Zallen.

Table of content

Table of figures

List of abbreviations

ADF: Actin depolymerizing factor

ANC: Actin-Nucleating Center

AP-1: Activator protein-1

Arp2/3: Actin related protein 2/3

Arm: Armadillo (*Drosophila* homogue of β-catenin)

Baz: Bazooka (*Drosophila* homologue of Par-3)

CA: Constitutively active

Cno: Canoe

Cor: Coracle

Crb: Crumbs

Dia: Diaphanous

Dlg: Disc Large

Dpp: Decapentaplegic

DGRC: *Drosophila* genomics resource center

DN: Dominant negative

DSHB: Developmental study hybridoma bank

Ed: Echinoid

EdΔC: Echinoid cytoplasmic tail deleted

EdΔP: Echinoid PDZ domain binding motif deleted

Ena: Enabled

F-actin: Filamentous actin

Fas III: Fasciclin III

Fmi: Flamingo

G-actin: Globular (monomeric) actin

GAP: GTPase activating protein

GDP: Guanosine diphosphate

GDI: Guanine nucleotide dissociation inhibitor

GEF: Guanine nucleotide exchange factor

GFP: Green fluorescent protein

(D)Grip: (*Drosophila*) Glutamate receptor interacting protein

GTP: Guanosine triphosphate

GTPase: Guanosine triphosphatase

JNK: Jun amino-terminal kinase

MARCM: Mosaic analysis with a repressible cell marker

MHC: Myosin II heavy chain

MLC: Myosin II light chain

PDZ: Psd-95, Disc Large, ZO-1

(p)MLC: (Phosphorylated) myosin II regulatory light chain

(D)MBS: (*Drosophila*) myosin II phosphatase

RFP: Red fluorescent protein

Rok: Rho kinase

Scrib: Scribbled

Std: Stardust

UAS: Upstream activating sequence

VASP: Vasodilator-stimulated phosphoprotein

Contributions of authors

My Ph.D. work is presented as two manuscript-based chapters.

Chapter 2 is a published manuscript:

Laplante, C. and Nilson, L.A. (2006). Differential expression of the adhesion molecule Echinoid drives epithelial morphogenesis in *Drosophila*. *Development* **133**, 3255-64.

I performed the experiments described in that chapter except for the isolation of the *echinoid*^{F72} allele, which Laura Nilson isolated. I collected and analyzed the data outlined in that manuscript. I prepared the figures and wrote the manuscript except for the discussion. Laura Nilson contributed guidance and revisions of the manuscript and wrote the discussion.

Chapter 3 is a manuscript in preparation:

I designed all the embryo experiments and Laura designed the MARCM clone experiment. I performed all the experiments described in that chapter. I collected and analyzed the data and wrote the manuscript. Laura Nilson provided guidance and revisions to the manuscript.

Abstract

Epithelial morphogenesis requires cell shape changes coordinated by local modulations of the actin cytoskeleton. We identify a role for Echinoid (Ed), a homophilic binding cell adhesion molecule, in the generation of contractile actomyosin cables that drive epithelial morphogenesis in both the *Drosophila* ovarian follicular epithelium and embryo. Specifically, we demonstrate that such contractile structures form at the interface of cells expressing and lacking Ed. Analysis of such interfaces generated by *ed* mutant follicle clones indicates that the juxtaposition of wild type and *ed* mutant cells results in the asymmetric localization of Ed in the wild type cell, which is sufficient to trigger the formation of an actomyosin cable at the clone interface. In wild type ovaries and embryos, specific cell types lack Ed, thus creating endogenous interfaces between cells with and without Ed; these interfaces display the same contractile cable as Ed interfaces created by *ed* mutant clones. In the ovary, this boundary lies between the two cell types of the dorsal appendage primordia. In the embryo, the absence of Ed from the amnioserosa during dorsal closure generates an Ed expression interface with the lateral epidermis, which coincides with the well-characterized actomyosin cable present in the epidermal cells at this interface. In both cases, elimination of Ed leads to the loss of the actomyosin cable causing subsequent defects in morphogenesis. Furthermore, we found that the asymmetric distribution of Ed in cells abutting Ed non-expressing cells is essential to polarize the actin regulators Diaphanous, Enabled and RhoGEF2 and downregulate the polarity protein Bazooka at the face of the cell where the actomyosin cable assembles. We conclude that the asymmetric distribution of Ed polarizes the actin cytoskeleton thus triggering the local and coordinated assembly of actomyosin cables at differential Ed interfaces. Disruption of the asymmetric distribution of Ed by ectopic expression of Ed transgenes in the amnioserosa maintains endogenous Ed uniformly distributed around the epidermal cells and prevents the formation of the actomyosin cable. This local modulation of the cytoskeleton at Ed expression interfaces may represent a general mechanism for promoting epithelial morphogenesis.

Résumé

La morphogénèse des épithelia requiert la coordination du movement des cellules par la modulation du cytosquelette d'actine dans chacune d'elles. Nous avons identifié un rôle pour Echinoid (Ed), une molecule d'adhésion à interaction homotypique, au cours de l'assemblage des câbles contractiles d'actomyosine qui promouvoient la morphogénèse des épithelia folliculaires ovariens et embryonaires chez la drosophile. Spéciquement, nous avons démontré que les structures d'actomyosine sont assemblées à l'interface entre des cellules qui expriment et d'autres qui n'expriment pas Ed. L'analyse de ces interfaces créées par la juxtaposition de cellules de type sauvage et de cellules mutantes pour *ed* causent la distribution assymmétrique de Ed dans la cellule de type sauvage ce qui promouvoit la formation du câble d'actomyosine à cette interface. De plus, certains types de cellules ovariennes et embryonaires n'expriment pas Ed de manière endogène créant ainsi des interfaces d'expression de Ed; ces interfaces assemblent aussi un câble contractile comme celles créées par les clones de cellules mutantes pour *ed*. Dans l'ovaire, ces interfaces se créent au contact des deux types de cellules qui formeront les appendices dorsaux. Dans l'embryon, l'absence de Ed dans l'amniosérose durant la fermeture dorsale génère une interface d'expression de Ed avec l'épiderme qui coincide avec la présence d'un câble d'actomyosine à cette interface. Dans chacun de ces cas, l'élimination de Ed cause la perte du câble menant à une morphogénèse aberrante. Nous avons aussi trouvé que la distribution assymmétrique de Ed dans les cellules adjacentes aux cellules qui n'expriment pas Ed est essentielle afin de polariser la localisation des régulateurs d'actine Diaphanous, Enabled et RhoGEF2 ainsi que la protéine de polarité Bazooka à la face de la cellule où le câble est assemblé. L'expression ectopique de transgènes codant pour Ed dans l'amniosérose maintien la distribution uniforme de Ed autour des cellules ce qui prévient la formation du câble d'actomyosine. Nous avons donc conclu que la distribution assymmétrique de Ed polarise le cytosquelette d'actine causant l'assemblage local de câble d'actomyosine aux interfaces d'expression differentielle de Ed. Cette modulation locale du cytosquelette pourrait représenter un méchanisme général promouvant la morphogénèse des épithelia.

Chapter 1: Literature review and introduction

1.1 Overview

Cells are capable of remarkable plasticity allowing them to move and change their shape to execute their specialized functions: white blood cells migrate to sites of infection and engulf cellular debris and pathogens; neurons elongate an axon that migrates in search of its target; mother cells divide their cytoplasm into two daughter cells during cytokinesis. Each of these processes represents the precise execution of a programme involving the temporal and localized remodeling of the cell's architecture. Cell shape changes and remodeling also occur in tissues where cells are attached to one another as a coherent unit. Examples of tissue movements include the multiple morphogenetic events that epithelia experience during development (for example, Schöck and Perrimon, 2002b). Indeed, the making of multicellular organisms involves the reorganization of flat sheets of cells, which bend to shape functional organs and give the characteristic body shape of organisms. These morphological programmes are highly regulated and properly executed according to the instructions received by cells during the determination of their fate. Furthermore, although mature epithelia give the misleading impression of being static and rigid entities, they reinstate programmes similar to those used during morphogenesis later during adult life in order to heal wounds or extrude dying cells (for example, Wood et al., 2002).

One of the most important contributors to cell shape is the actin cytoskeleton; F-actin anchored to the plasma membrane, cortical actin, builds a rigid support that shapes

the cell. However, when dynamically dissolved and rebuilt at precise subcellular locations, it provides a driving force to cell motility (Pollard and Borisy, 2003). In addition to altering their cortical actin, single cells migrate by locally modulating their adhesion to the substratum, which anchors cells to an extracellular matrix during movements. The remodeling of tissues represents a complex endeavour as cells must alter their cytoskeleton in a coordinated fashion while maintaining cell-cell attachment and thus the coherence of the tissue. The members of the family of Rho small GTPases, master-switches that cycle between active and inactive states, are at the nexus of cytoskeleton and cell adhesion regulation and act to regulate morphogenesis (Jaffe and Hall, 2005). The activation and inactivation of the Rho small GTPases at precise subcellular locations generate localized environments where actin and cell junctions are assembled or disassembled. In a migrating cell or group of cells, such localized regulation directs cellular movement so that the actin cytoskeleton and cellular adhesion are controlled differently at the leading edge than at the trailing end.

The formation of a contractile multicellular actomyosin cable at the leading edge provides tension during the concerted migration of tissues, epithelial wound closure and apoptotic cell extrusion. Although studies have so far enumerated multiple molecules involved in the control of the actin cytoskeleton and cell adhesion to assemble actomyosin cables, we still have to determine how these building blocks are polarized within each cell to form the cable only at the leading edge of the migrating cells. Moreover, it is still unclear how these changes in the actin cytoskeleton are coordinated across a tissue during morphogenesis. It is likely that the answers to these quandaries

reside at least partly in the control of cell adhesion properties and thus the differential expression of adhesion molecules between neighbouring cells. Indeed, cellular adhesion can relay information about neighbouring cells thereby influencing the actin cytoskeleton and the cytoplasm.

Most of our current knowledge about the regulation of actin cytoskeleton and adhesion comes from studies done on cultured cells. While these malleable model systems are extremely effective at identifying and dissecting the biochemical functions of molecules involved in cell adhesion and actin regulation, they can lead to contradicting results when used to understand their molecular and cellular functions because of variations between cell types and growth conditions (for example, Trichet et al., 2008; Vasioukhin et al., 2000). The use of simple genetic organisms like *Drosophila* can complement the work done in cell culture by studying the function of genes in the context of a developmental process in an intact tissue. As my work focused on the formation of multicellular actomyosin cables, I used the formation of the dorsal appendages producing tubes during oogenesis and the closure of a dorsal epithelial gap during embryogenesis as platforms for the study of different actin regulators. To complement this work, I used genetic tools available in *Drosophila* to generate ectopic actomyosin cables in epithelia, thus providing a malleable model system and an alternative to the more complex events that occur during morphogenesis.

1.2 The actin cytoskeleton: the powerhouse of cellular tension

1.2.1 Actin polymerizes into filaments

The actin cytoskeleton is one of the major contributors of morphogenesis: it determines the shape of cells, it continuously remodels in response to environmental stimuli, and it is composed not only of actin but also a multitude of regulatory actin binding proteins. Actin is a remarkable protein with the capability to polymerize spontaneously into thin, flexible filaments that adopt the shape of interlaced double helices. Each actin monomer (also known as globular or G-actin) exhibits an intrinsic polarity and can mediate head-to-tail interactions with two other actin monomers to form actin filaments (also known as filamentous or F-actin) (Chhabra and Higgs, 2007; Pollard et al., 2000). Consequently, since all the actin monomers are oriented in the same direction, actin filaments have a distinct polarity with distinguishable ends. The polarity of F-actin can be determined by incubating filaments with the S1 subunit of myosin II, which binds to actin adopting a chevron shape along the actin filament. Actin filaments decorated with S1 subunits display distinguishable ends according to the orientation of the chevrons: the pointed end (plus end) and the barbed end (minus end) (Pollard, 1986). Using a combination of *in vitro* polymerization and electron microscopy, it was determined that the barbed end, or plus end, of the filament elongates almost 10 times faster than the opposite pointed, or minus, end (Pollard, 1986).

The polarity of actin filaments is therefore central to its polymerization and depolymerization properties and by extension to its function *in vivo*. The

polymerization of F-actin has been extensively studied *in vitro* as actin monomers can conveniently self-assemble into filaments (Chhabra and Higgs, 2007; Pollard et al., 2000). From such work we know that the first step in making a filament consists in the formation of small oligomers of three to four actin subunits that act as a nucleus or seed. This process is unfavorable as small oligomers are very unstable and tend to disassemble before they can be elongated. However, once produced, these short oligomers act as seeds that rapidly polymerize by the addition of G-actin to both ends of the growing filament, albeit with higher efficiency at the barbed end. *In vitro*, the polymerization of F-actin is linked to the concentration of available actin monomers in solution. As filaments grow, the concentration of G-actin monomers in solution decreases until equilibrium is reached between free G- and F-actin.

Another fascinating characteristic of actin is that as filaments polymerize, the actin molecules making the filament activate a self-destruction mechanism. This feature is linked to the ATPase enzymatic function of actin. After ATP-bound G-actin (ATP-actin) is polymerized into a filament, its ATPase function increases over 40,000 fold; ATP is hydrolyzed to ADP and inorganic phosphate (P_i) and then P_i is slowly released from actin (Fujiwara et al., 2007). But actin doesn't need the energy from ATP hydrolysis to drive polymerization as ADP-actin and actin bound to a non-hydrolyzable form of ATP can still polymerize *in vitro* (Cooke, 1975a; Cooke, 1975b; Pollard, 1986). Instead, the hydrolysis of ATP destabilizes the filament by increasing the rate of depolymerization. This conclusion was drawn from a series of experiments that measured the rate of association and dissociation of ATP-, ADP/P_i- or ADP-bound G-

actin (Fujiwara et al., 2007; Pollard, 1986). It was found that ATP-actin and ADP/Pi-actin preferentially associate into filaments while ADP-actin preferentially dissociates from filaments. Moveover, the reloading of actin with fresh ATP has never been observed to occur within filaments, suggesting that this exchange must occur only when actin is in a monomeric state (Fujiwara et al., 2007). Following such findings, it was suggested that ATP hydrolysis prepares the filament for disassembly thus providing a built-in mechanism to destabilize F-actin.

To recapitulate, the barbed end of F-actin elongates faster than the pointed end, ATP-actin preferentially associates to growing filaments while ADP-actin preferentially dissociates and hydrolysis of the nucleotide occurs as the filament matures. It ensues that the barbed end is the preferred site for the elongation of the filament via the addition of ATP-actin and the pointed end is the preferred site of ADP-actin dissociation. Moreover, once the equilibrium of free G-actin and F-actin in solution has been reached, there is no net gain or loss of F-actin in solution. Put together, those parameters illustrate a phenomenon termed treadmilling, during which an actin filament of constant length contains ATP-actin at the barbed, ADP/P_i-actin in the middle and ADP-actin at the pointed end. Newly inserted monomers are displaced within the filaments toward the pointed end as new monomers are inserted at the barbed end. They hydrolyze their ATP, which enhances their rate of dissociation from the filament from the pointed end. Although treadmilling has been observed *in vitro* (Fujiwara et al., 2002), it is not known whether it can provide the force necessary for cell shape changes and movements *in vivo*.

1.2.2 Actin nucleators catalyze the formation of new actin filaments

As mentioned earlier, *de novo* formation of actin filaments requires the assembly of small oligomers of three to four actin monomers; this step is very unfavorable due to the instability of such short aggregates (Sept and McCammon, 2001). How do cells overcome this rate-limiting step when rapid polymerization is required? Certain actin binding proteins such as formins, the Actin Related Protein 2/3 (Arp2/3) complex, and Spire hasten the rate of nucleation of G-actin thus jump-starting the assembly of F-actin (Kerkhoff, 2006; Pollard, 2007). Each of these factors is tightly regulated both by the control of its subcellular localization and interaction with activating proteins to prevent their spontaneous activation. For the purpose of this work, we will focus on the role of Diaphanous type formins and the Arp2/3 complex.

Formins bind G-actin and generate unbranched actin filaments that participate in a variety of actin structures such as actomyosin cables like the cytokinetic contractile ring and filopodia (Goode and Eck, 2007; Pollard, 2007). Formins of the Diaphanous family contain four important domains essential for their function: Diaphanous Autoregulatory Domain (DAD), Rho binding domain (RBD) and Formin Homology type 1 and 2 (FH1 and FH2) domains. Two Formin molecules dimerize into the shape of a doughnut via interactions between their formin homology domain (FH) 2 domains. The nucleating ability of formins lies in their structure: the hole of the Formin doughnut is large enough to envelop about two actin monomers and this confirmation is thought to stabilize the small aggregates. In addition, the FH1 domain contains multiple

polyproline sequences that interact with Profilin. As Profilin can bind simultaneously to both the FH1 domain and ATP-actin, it is thought this interaction concentrates ATP-actin close to the barbed end of the elongating filament and therefore formins have the potential to enhance the rate of actin polymerization (Pollard, 2007).

Such an efficient polymerizing factor must be tightly regulated to prevent uncontrolled assembly of filaments. Indeed, formins can switch between an inactive and an active conformation depending on the state of an intramolecular interaction. The DAD at the carboxy-terminus of formins interacts with a sequence in its amino-terminus keeping it in a closed, inactive form. The DAD binding site at the amino-terminus overlaps with RBD and the binding of active Rho-GTP to the RBD competes away the interaction with DAD thus opening the Formin into an active conformation that can dimerize (Pollard, 2007).

Another well-characterized actin-nucleating factor is the Arp2/3 complex. The Arp2/3 complex promotes the formation of branched F-actin, which is essential during endocytosis, intracellular movements of vesicles and the formation of lamellipodia at the leading edge of migrating cells (Pollard, 2007). The Arp2/3 complex, composed of 7 ARP proteins (named ARP 1 to 7), can generate a branch of F-actin at the side of an existing mother filament by tethering the pointed end of a new filament to the pre-existing branch and exposing the free barbed end for elongation. As for formins, the activity of the ARP2/3 complex is tightly regulated. The Arp2/3 complex is inherently

inactive and requires the cooperation of nucleation promoting factors (e.g. WASP and Scar/WAVE), G-actin, and pre-existing F-actin for its activation.

1.2.3 G-actin binding proteins regulate actin polymerization

The self-assembly of F-actin is convenient for studying actin dynamics *in vitro* but in a cell, the uncontrolled polymerization of F-actin would have disastrous repercussions on basic cellular functions. Although the high intracellular concentration of G-actin is sufficient to promote spontaneous polymerization, cells maintain a large amount of unpolymerized G-actin (Pollard et al., 2000). This pool of free G-actin is crucial for the rapid turnover of actin filaments during a variety of cell movements and its regulation is therefore relevant to the control of morphogenesis. To control this pool of available G-actin, and thus the formation of F-actin, cells use a multitude of actin interacting proteins each of which can influence polymerization either by promoting monomer disassembly or assembly. Actin binding proteins influence the cytoskeleton in various ways: they can sever filaments, sequester G-actin monomers in the cytoplasm preventing them from self-assembling or promote the assembly of filaments. There are hundreds of actin binding proteins but here I will describe the characteristics of two of them: Cofilin and Profilin.

One of the key proteins responsible for actin filament disassembly is Cofilin (cosediments with filamentous actin; encoded by the gene *twinstar* in *Drosophila*). Cofilin belongs to a family of Actin Depolymerizing Factors (ADF), which bind to ADP-actin filaments and promotes their disassembly. Cofilin has two known modes of

action: it enhances the rate of dissociation of ADP-actin from the pointed end and can sever actin filaments (Paavilainen et al., 2004). The fate of the severed filaments depends on the local concentration of G-actin; at high concentration, severed filaments preferentially polymerize while at low G-actin concentration, they preferentially depolymerize and G-actin is sequestered by Cofilin. Therefore, although Cofilin depolymerizes F-actin, it actually can promote dynamic polymerization of filaments by releasing ADP-actin and making them available for another round of polymerization. The phosphorylation of Cofilin prevents its binding to both G- and F-actin and is therefore referred to as the inactive state. Different kinases phosphorylate Cofilin including LIM kinases regulated by Rho small GTPases while phosphatases including Slingshot can activate Cofilin. Alternatively, Cofilin can be maintained inactive at the membrane by binding to phosphatidylinositol-4, 5-bisphosphate (PIP_2). These two regulatory mechanisms occur predominantly at the membrane suggesting that Cofilin is inhibited at the interface of F-actin and the membrane where actin polymerization is predominant but active deeper in the cells where actin monomers disassemble to be recycled.

Another cytosolic protein, Profilin (encoded by the gene *chickadee* in *Drosophila*) binds actin monomers in a stable 1:1 complex and promotes the polymerization of F-actin (Paavilainen et al., 2004; Pollard, 2007). One way Profilin enhances filament assembly is by binding to ADP-actin monomers in a conformation that allows the exchange of ADP for ATP therefore enhancing polymerization. The action of Profilin is enhanced by the presence of Cofilin as Cofilin can remove ADP-

actin from the pointed end of an aging filament and hand it to Profilin to exchange the nucleotide and recycle the monomer to the growing barbed end. Moreover, as mentioned earlier, Profilin can interact with both ATP-actin and the FH1 domain of Formins therefore bringing actin monomers to the barbed end of a growing actin filament (Pollard, 2007).

1.2.4 Myosin II and contractile force

Contractile force is essential to bend tissues and divide cells during cytokinesis. How do cells generate the tension necessary for such processes? Myosins are motor proteins that bind actin and use the chemical energy generated by successive cycles of ATP hydrolysis to move in a unidirectional fashion along filaments (Eddinger and Meer, 2007). Myosin II (named myosin II for its two heads) was the first myosin to be discovered and is a hexameric macromolecule composed of two heavy chains and 4 light chains. Each heavy chain of myosin II comprises three regions: a head, a neck and a tail. The N-terminal head region contains the motor domain that binds to both actin and ATP and hydrolyzes ATP for energy. The neck region of the protein binds to two light chains: an essential and a regulatory light chain. Finally the long coiled-coil domain of the tail of the heavy chain mediates the spontaneous dimerization between heavy chains. The coiled-coil tails can further bundle the tails of myosin II molecules resulting in the formation of large bipolar filaments with myosin heads oriented in opposite direction at the two ends of the myosin filament. This configuration permits myosin II to bind to antiparallel actin filaments and slide them toward each other upon activation thus causing constriction.

The actin cytoskeleton is enriched just underneath the plasma membrane and interacts with it at specific sites thus providing a rigid cortex that shapes the cell. The actin motor protein non-muscle myosin II binds antiparallel actin filaments and cause them to slide in opposite directions toward each other resulting in the overall constriction of filaments (Clark et al., 2007). As the filaments are anchored to the plasma membrane via interactions with membrane-bound proteins, this constriction of the actin filaments causes the plasma membrane to adopt different shapes and is essential for cell movements (for example, Fox and Peifer, 2007).

The regulation of non-muscle myosin II (thereafter referred to as myosin II) mostly relies on the phosphorylation state of the regulatory myosin II light chain (MLC; encoded by *spaghetti squash* in *Drosophila*) (Karess et al., 1991). Phosphorylation of *Drosophila* MLC on Threonine 20 and Serine 21 increases the actin-activated ATPase activity and thus the contraction of actin filaments (Jordan and Karess, 1997). Different kinases phosphorylate MLC including myosin light chain kinase (MLCK) and Rho kinase (Rok; DRok in *Drosophila*) while the myosin II phosphatase Myosin Binding Subunit (MBS; DMBS in *Drosophila*) dephosphorylates MLC. These proteins are further regulated in part by the Rho small GTPase pathway (Adelstein et al., 1978; Amano et al., 1996; Kimura et al., 1996).

1.2.5 Actomyosin cables

A common actin structure that participates in a variety of single and multicellular movements is the actomyosin cable (Bement, 2002; Darenfed and Mandato, 2005). Actomyosin cables, as their name suggests, are bundles of F-actin crossed-linked by myosin II and thus act like contractile "purse strings" upon activation of myosin II. Single cells assemble an actomyosin cable at their equator during cytokinesis and the tension generated by the constriction of this cable splits the cell's cytoplasm into two daughter cells (for example, Hickson et al., 2006; Wu et al., 2003). Multicellular actomyosin cables are cables assembled within neighbouring cells of a tissue. Each cell that participates to the elaboration of the multicellular actomyosin cable coordinates the assembly of a piece of actomyosin at one of its faces. For example, in a wounded tissue, the cells at the wound edge coordinate the assembly of a piece of actomyosin cable at the edge of the wound so that together all the segments of actomyosin in the different cells generate a multicellular cable that appears continuous.

Much of our knowledge of multicellular cables comes from work on wound healing and developmental processes that involve the closure of epithelial gaps as well as from the extrapolation of our knowledge of single cell actomyosin rings. Although we still have a lot to learn about actomyosin cables, it appears that a common mechanism clearly exists for the assembly of both single and multicellular cables; they both require the activation of Rho small GTPases, actin polymerization and myosin II-driven contraction. Multicellular cables involve an extra level of complexity; each of the cells participating to the elaboration of the multicellular cable must coordinate its

cytoskeleton in the same polarized fashion and the tension generated by the contractile structure must be coupled among the cells potentially via cellular junctions. It is still unclear whether the trigger to produce a multicellular actomyosin cable is communicated between neighbouring cells or whether each cell independently receives this inductive signal from an external source. Furthermore, it is still unclear how each of the cells that participate to the formation of the multicellular cable anchors its portion of cable at its plasma membrane.

1.3 Cell-cell adhesion and cortical actin cytoskeleton

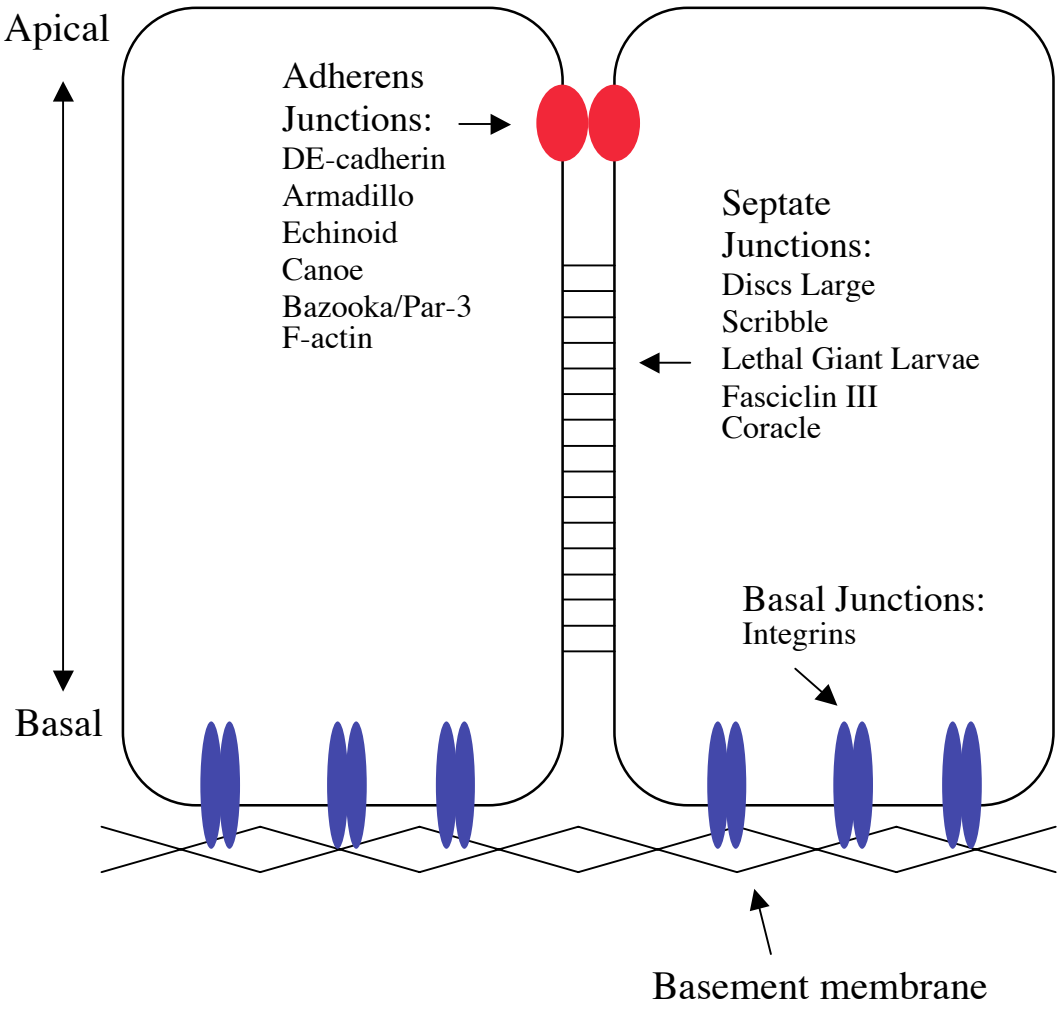
The cortical network of actin cytoskeleton influences cell shape by interacting with the plasma membrane. Indeed, the organization of F-actin into bundles or meshworks and their interaction with the membrane induce cells to adopt varied shapes; meshworks of branched actin in lamellipodia push the membrane of migrating cells while bundles of unbranched actin in the cytokinetic actomyosin cable pull in the cleavage furrow in dividing cells (Pollard, 2007). Different proteins can link actin to the plasma membrane; most relevant to this work are the cell adhesion junctions, specialized protein complexes that link cells to each other or to the substratum.

Epithelial tissues are made of coherent, adhering cells and adopt an apical-basal polarity, which divides the tissue into functional domains. Apical and basolateral domains display distinct collections of membrane-bound proteins that participate in different functions of the cell, and by extension, of the tissue. The apical domain lines the outside of tissues or the lumen of epithelial tubes therefore participating in exchange

with the milieu, while the basolateral domain connects cells to each other and to the basement membrane. Invertebrates, like *Drosophila*, exhibit three distinct junctional complexes: adherens junctions, septate junctions and basal junctions (Muller, 2000). Unlike vertebrates, invertebrates lack tight junctions, which are responsible for providing a permeability barrier to the passage of molecules between compartments; instead, invertebrate septate junctions perform the role of permeability barrier (Furuse and Tsukita, 2006). The junctional complexes are arranged in a polarized fashion with the adherens junctions at the apical-most portion of the lateral domain followed by the septate junctions along the basolateral domain (Figure 1.1). The basal junctions are distributed over the basal portion of the cell and make contact with the basement membrane mainly via the interaction between integrin adhesion molecules and the extracellular matrix. Adherens junctions are most relevant to my work and following is a summary of their characteristics.

Figure 1.1 Epithelial polarity in *Drosophila*.

Schematic representation of epithelial cells illustrating the organization of adherens junctions, septate junctions and basal junctions. Examples of proteins enriched at each junctional complex are listed.


Apical
Basal
Adherens
Junctions:
DE-cadherin
Armadillo
Echinoid
Canoe
Bazooka/Par-3
F-actin
Septate
Junctions:
Discs Large
Scribble
Lethal Giant Larvae
Fasciclin III
Coracle
Basal Junctions:
Integrins
Basement membrane

Adherens junctions are sites of cell-cell adhesion characterized by the enrichment of adhesion molecules, their cytosolic binding partners and F-actin organized into a circumferential belt. Epithelial cadherin (E-cadherin in mammals and DE-cadherin in *Drosophila*) is one of the best-characterized adhesion molecules enriched in adherens junctions and is instrumental to the maintenance of contact between epithelial cells; the removal of E-cadherin causes cells to detach, lose their columnar shape and instead adopt a round shape (C.L. unpublished observations, Muller, 2000; Oda et al., 1997). Cadherins promote calcium-dependent adhesion (hence their name) between neighbouring cells (Halbleib and Nelson, 2006). Like other classical cadherins, E-cadherin molecules from neighbouring cells engage their extracellular domain in homophilic interactions and their cytoplasmic tails function as docking sites for cytosolic proteins notably β-catenin (also known as Armadillo (Arm) in *Drosophila*), which in turn binds to α-catenin. As α-catenin can bind to both β-catenin and F-actin, it was thought that it anchored cadherins to the cortical actin network by directly linking them together (Hinck et al., 1994; Pokutta et al., 2002; Pokutta and Weis, 2000; Rimm et al., 1995). Contrary to this classical model, recent studies have shown that α-catenin can bind either to β-catenin or to F-actin but never to both simultaneously, implying that this selective interaction depends on the conformation of α-catenin (Drees et al., 2005; Yamada et al., 2005). Indeed, α-catenin can adopt either of two conformations: as a monomer it binds strongly to β-catenin, as a homodimer it interacts preferentially with F-actin. Therefore, α-catenin cannot directly link actin to the E-cadherin/β-catenin complex. In spite of those results, cadherins may still be anchored to the actin cytoskeleton directly albeit via proteins other than α-catenin. These findings remind us

that although the direct interaction between E-cadherin and the actin cytoskeleton appears logical to provide the support for cell-cell adhesion, the nature of this interaction is likely to be much more dynamic than previously thought.

The organization of the actin cytoskeleton in a circumferential belt at the level of the adherens junctions is nonetheless important to cell-cell adhesion. Indeed, the actin cytoskeleton is essential for the establishment of stable E-cadherin mediated adhesion and by extension stable cell-cell contacts (Vasioukhin and Fuchs, 2001). During early *Drosophila* embryogenesis, the stabilization of nascent adherens junctions depends on the role of Bitesize, a synaptotagmin-like protein, which participates in a mechanism that organizes the actin cytoskeleton (Pilot et al., 2006). In the absence of Bitesize, the actin cytoskeleton fails to form a continuous network at the cortex and spots of DE-cadherin are recruited to the membrane but are not stabilized into a uniform belt. Bitesize is recruited to the membrane by interaction with both phosphatidylinositol-4,5-bisphosphate (PIP_2) and the polarity protein Par-3 (also known as Bazooka (Baz) in *Drosophila*). Once at the plasma membrane, Bitesize recruits Moesin, a FERM domain containing protein, which interacts with both the plasma membrane and actin thereby participating in the elaboration of the actin network of the nascent adherens junctions (Pilot et al., 2006).

This enrichment of F-actin at the cortex where nascent adherens junctions are stabilized occurs at least partly by local polymerization. Indeed, the nucleation factors Arp2/3 complex and formin are enriched at adherens junctions suggesting a role for both

branched and unbranched F-actin for the formation and/or the stabilization of adherens junctions. Work on mammalian cultured cells has shown that E-cadherin engagement recruits the Arp2/3 complex to zones of nascent cell-cell contacts thus influencing the site of actin assembly (Kovacs et al., 2002b; Verma et al., 2004). Furthermore, during the formation of epithelial sheets from cultured cells, α-catenin recruits Formin-1 to sites of nascent adherens junctions thus participating in the assembly of linear actin cables (Kobielak and Fuchs, 2004; Kobielak et al., 2004).

The F-actin interacting protein Enabled/VASP (Ena) is also enriched at nascent junctions in mammalian tissues (Vasioukhin et al., 2000). Ena is a regulator of actin but its molecular function remains debatable. In some work, it was shown that Ena enhances the rate of actin polymerization and protects the barbed end of filaments from the binding of capping proteins that terminate polymerization and destabilize filaments (Barzik et al., 2005; Plastino et al., 2004a; Plastino et al., 2004b; Samarin et al., 2003). However, contradicting results were obtained under different conditions (Barzik et al., 2005; Bear et al., 2000; Bear et al., 2002; Samarin et al., 2003). Yet, so far all agree to say that Ena participates in the remodeling of F-actin and inhibits the formation of branched actin by the Arp2/3 complex. The recruitment of Ena to adherens junctions might therefore be involved in the switch from branched F-actin required in the formation of nascent adherens junction to the unbranched type required for their stabilization and strengthening.

1.3.1 Adherens junction and actomyosin cables

As described above, multicellular actomyosin cables are made of actin filaments bound by myosin II that assemble concomitantly within neighbouring cells thus appearing to be continuous (Bement, 2002). It is still not clear how pieces of actomyosin cable are anchored at the membrane between cells that share the multicellular cable. A sensible hypothesis is that the multicellular actomyosin cable is anchored at the adherens junctions. A study performed on wounded embryonic corneal epithelia suggests that adherens junctions are involved in this attachment (Danjo and Gipson, 1998). When embryonic mouse corneal epithelia are wounded, they assemble an actomyosin cable at the edge of the wound. This actomyosin cable colocalizes with adherens junctions and therefore appears to be linked by adherens junctions at the interface of the cells that share the multicellular cable at the wound edge. Danjo and colleague found that the actomyosin cable is lost upon treatment with an antibody that destabilizes the function of E-cadherin and instead the leading edge of the wound forms large projections reminiscent of lamellipodia. In the interpretation of their results, Danjo and colleague stated that adherens junctions might anchor the actomyosin cable at neighbouring cell interfaces and in their absence, the cable is lost due to lack of anchoring. However, since the publication of this work, it has been shown that adherens junctions are not merely performing the role of intercellular glue but also participate in cellular signaling. It is therefore not clear whether the loss of the actomyosin cable is caused by the removal of their adherens junction anchor or whether the loss of adherens junctions triggers changes in the cytoskeleton, indirectly for example via changes in the regulation of Rho small GTPases.

1.4 Rho small GTPases: master regulators of cell shape and adhesion

The execution of morphogenetic programmes requires the tight control of the actin cytoskeleton and the coordination and modulation of cellular adhesion. Members of the Rho family of small GTPases, namely Rho, Rac and Cdc42, are closely involved in the regulation of the building blocks that sculpt the architecture of tissues. Indeed, multiple studies have implicated the role of Rho small GTPases in the control of the actin cytoskeleton during the execution of cell movements, morphogenesis and cellular adhesion (Buchsbaum, 2007; Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005; Van Aelst and Symons, 2002). These regulatory GTPases respond to extracellular stimuli and act as molecular switches that cycle between inactive GDP-bound and active GTP-bound states. The complexity of their function relies on a simple biochemical mechanism: switched on, Rho small GTPases bind to and activate their targets, often by opening an autoinhibiting intramolecular interaction in the inactive target protein. There are three classes of regulatory proteins that affect the activation state of Rho small GTPases: activating guanine nucleotide exchange factors (GEFs) promote exchange of GDP for GTP; inactivating GTPase activating proteins (GAPs) enhance the intrinsic GTP hydrolysis activity; and guanine nucleotide dissociation inhibitors (GDIs), which block spontaneous activation.

Much of our knowledge of Rho small GTPases comes from the ectopic expression of dominant negative (DN; cannot release GDP) and constitutively active (CA; cannot hydrolyze GTP) constructs. Rac-CA induces the assembly of branched actin protrusions (lamellipodia), Rho-CA leads to the assembly of unbranched actin and

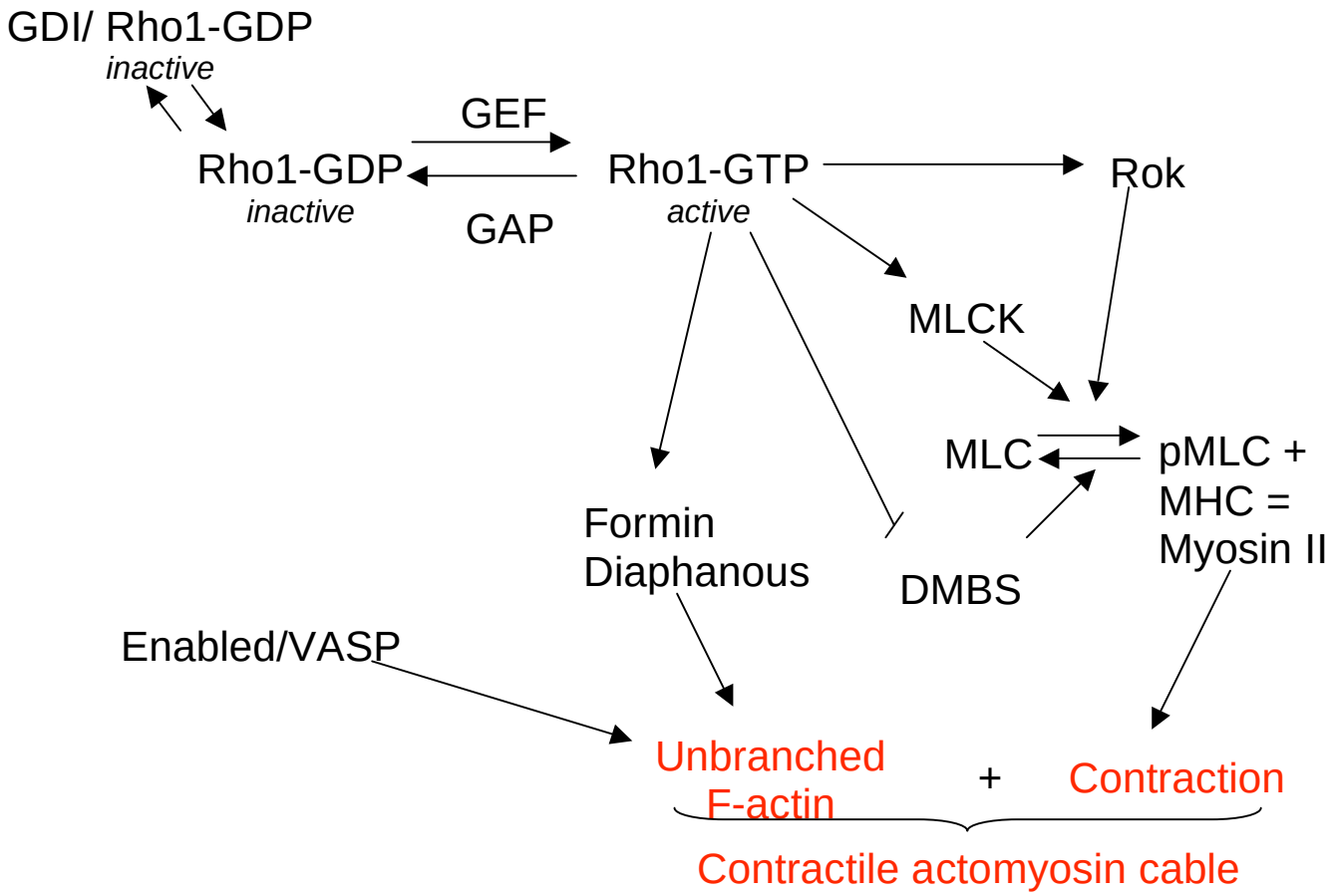
myosin contractile filaments (stress fibers) and Cdc42-CA causes the formation of thin actin-rich protrusions (filopodia) (Nobes and Hall, 1995; Ridley and Hall, 1992; Ridley et al., 1992). Such initial studies on those three classes of Rho small GTPases identified their pivotal role in the regulation of the actin cytoskeleton.

1.4.1 Rho small GTPases regulate the actin cytoskeleton

Rho regulates the actin cytoskeleton by promoting the polymerization of unbranched F-actin and by activating myosin II. The Diaphanous-type formins promote the assembly of unbranched F-actin. As discussed above (section 1.2.2), formins normally adopt a closed inactive confirmation via intramolecular interaction between their carboxy-terminus DAD and amino-terminus RBD (Pollard, 2007). Upon stimulation, Rho-GTP binds to the RBD thereby displacing the intramolecular interaction with the DAD and opening the molecule into an active conformation, which can homodimerize and nucleate F-actin. Additionally, Rho-GTP promotes the constriction of actin filaments via its downstream target Rho kinase (Rok). Rok activates myosin II via three distinct routes: by directly phosphorylating and activating MLC, by phosphorylating and activating myosin light chain kinase (MLCK), which directly phosphorylates MLC, and finally by phosphorylating and inactivating MBS (Adelstein et al., 1978; Amano et al., 1996; Kimura et al., 1996). All together these different alternatives give rise to the same general outcome: the activation of MLC and thereby the contraction of actin filaments. This pathway is pivotal to the formation of stress fibers and contractile actomyosin cables (Figure 1.2).

Figure 1.2. The Rho1 signaling pathway.

Diagram of the summarized Rho1 signaling pathway in the formation of contractile actin structures. GAP: GTPase activating protein, GEF: guanine nucleotide exchange factor, GDI: guanine nucleotide dissociation inhibitor, Rok: Rho kinase, MLC: myosin II light chain, MHC: myosin II heavy chain, MLCK: myosin II light chain kinase, DMBS: *Drosophila* myosin II phosphatase, VASP: Vasodilator-stimulated phosphoprotein.


GDI/ Rho1-GDP
inactive
GEF
Rho1-GDP
inactive
GAP
Rho1-GTP
active
Rok
MLCK
MLC
pMLC +
MHC =
Myosin II
Formin
Diaphanous
DMBS
Enabled/VASP
Unbranched
F-actin
+
Contraction
Contractile actomyosin cable

Rac and Cdc42 alter the actin cytoskeleton via the activation of the actin nucleator Arp2/3 complex (Pollard, 2007; Takenawa and Miki, 2001). As described above, the Arp2/3 complex exists mainly in an inactive conformation and requires the role of the activator WASP/Scar to be switched on. *In vitro*, Cdc42-GTP binds to and activates WASP via the relief of an intramolecular autoinhibitory interaction within the WASP protein thus exposing its Arp2/3 activation site (Carlier et al., 1999; Egile et al., 1999; Machesky et al., 1999; Rohatgi et al., 1999). Rac mediates the activation of Arp2/3 by indirectly activating another Arp2/3 activator named WAVE (Sasaki et al., 2000). The resulting activation of the Arp2/3 complex by either Rac or Cdc42 leads to the formation of branched actin filaments. Interestingly however, Rac and Cdc42 each mediate the formation of very distinct types of actin protrusions: lamellipodia by Rac and filopodia by Cdc42. As filopodia are bundles of unbranched F-actin, this indicates that the activation of Cdc42 ultimately results in the formation of unbranched bundles of F-actin from branched F-actin networks made by Arp2/3.

1.4.2 Rho small GTPases and cell adhesion

Although Rho small GTPases have been studied extensively for their action on the actin cytoskeleton, they also contribute to morphogenesis by regulating cellular adhesion and polarity. For example, the inhibition of Rho1 causes the removal of cadherins from junctions suggesting that Rho plays a role in adherens junction formation by stimulating cadherin clustering. This effect has been observed in cultured cells expressing Rho-DN, cells treated with the Rho inhibitor C3 transferase and also in *Drosophila* embryos expressing ectopic Rho1-DN (Bloor and Kiehart, 2002; Braga et

al., 1999; Braga et al., 1997; Takaishi et al., 1997). However, *Drosophila* embryos mutant for *rho1* do not exhibit loss of adherens junctions and loss of cell contacts (Magie et al., 1999). This discrepancy could be explained by the fact that *rho1* mutant embryos do not completely lack Rho1 function as the complete lack of Rho1 arrests development during oogenesis. Alternatively, as Rho small GTPases are very similar in their primary sequences and share some of the same GEFs and GAPs, dominant mutations might affect other GTPases therefore causing strong pleiotropic effects.

Rac function is essential for the accumulation of F-actin at adherens junctions and to promote the stabilization and maturation of nascent adherens junctions. In cultured mammalian cells, Rac is essential for the establishment of adherens junctions (Braga et al., 1999; Braga et al., 1997; Takaishi et al., 1997). Moreover, expression of Rac1-DN in the *Drosophila* wing imaginal disc inhibits the assembly of the actin belt at the level of the adherens junctions (Eaton et al., 1995). In mammalian cells, E-cadherin mediated adhesion recruits Rac to junctions, which in turns stabilizes actin assembly (Kovacs et al., 2002a; Nakagawa et al., 2001; Noren et al., 2001). As Rac activates the Arp2/3 complex, it may mediate the reorganization of the actin cytoskeleton via the Arp2/3 complex, which in turn is necessary to stabilize cadherin at cell-cell contact sites.

Cdc42 influences the polarization of cultured epithelial cells by regulating the polarity complex Par-3/Par-6/aPKC (Joberty et al., 2000; Lin et al., 2000; Qiu et al., 2000). This polarity complex is recruited to the apical-most portion of cell-cell contact

during the initial stages of apical-basal polarization. Work in *Drosophila* suggests that Cdc42 interacts with Par-6, which is important for the activation of aPKC and the subsequent phosphorylation of Par-3 and this mechanism is crucial for the proper establishment of apical-basal asymmetry (Hutterer et al., 2004). Unfortunately, the role of that complex it is not fully understood but its distribution is tightly linked to the proper polarization of epithelial cells.

1.4.3 Regulation of Rho small GTPases

The Rho small GTPases function in a broad variety of cellular events and understanding their regulation is fundamental to shed light on their intricate roles. The regulation of Rho small GTPases is controlled by GEFs, GAPs and GDIs. Moreover, the subcellular localization of the GTPases also influences their activity and is crucial to direct their function to the desired subcellular position. As the modulation of the actin cytoskeleton often occurs at the cell cortex, it appears likely that Rho small GTPases are recruited to the plasma membrane for their activation. Different pieces of evidence suggest that this is the case. Rho small GTPases are modified by the addition of a lipophilic prenyl group that interacts with the plasma membrane and this localization is essential for their activity (Cohen et al., 2000). GDIs bind to prenylated GDP-bound GTPases and promote their translocation from the plasma membrane to the cytosol, therefore keeping them away from their site of activity (Mondal et al., 2000). Additionally, most GEFs contain a Pleckstrin Homology (PH) domain, which interacts with phospholipid and localizes GEFs to the plasma membrane thereby targeting the activation of the GTPases to the cell cortex (Buchsbaum, 2007). And finally, certain

GEFs, such as *Drosophila* RhoGEF2, contain a PDZ (Psd-95, Disc Large, ZO-1) protein-protein interaction domain known to interact with short sequences in the very end of the cytoplasmic tail of transmembrane proteins named PDZ domain binding motifs. Therefore, migrating cells must use a complex interplay of signals to compartmentalize the activity of Rho small GTPases according to particular subcellular locations, probably via the local enrichment of GEFs, GAPs and GDIs.

1.5 Morphogenesis, cellular contacts and actin cytoskeleton

The shaping of multicellular organisms from a single cell zygote is the combined result of cell division and tissue reorganization, which involves a complex variety of cell shape changes. In epithelial tissues, cells adopt a roughly hexagonal shape and consequently each cell vertex usually contacts only three neighbours at a given time (Figure 1.3A) (Carthew, 2005; Lecuit and Lenne, 2007). This organization is dictated by the properties of adherens junctions and is adopted spontaneously during the establishment of polarity in epithelial cells. What compels cells to spontaneously adopt such shape? If you observe the soap bubbles that form on the surface of your dishwater for example, you'll notice that bubbles also pack as hexagons. In fact, soap bubbles adopt this shape when they contact one another in order to minimize surface tension created by the contact with air. From this resemblance, it is thought that epithelial cells pack into hexagons in order to adopt a thermodynamically favourable state (Carthew, 2005). During morphogenesis however, cells spend energy to alter their shapes, which in turn rearranges tissues into their final structures. There are different common cellular rearrangements epithelial cells utilize during development including cell shape changes,

cellular fusion and cellular movement (Schöck and Perrimon, 2002b). During invagination and evagination, epithelial cells locally constrict their apical domains via the reorganization of their cytoskeleton resulting in remodeling of epithelial shape as a whole. New cell-cell adhesion can be created during the fusion of epithelial sheets, while during cell intercalation cells change their neighbours, which requires the breaking down and creation of new cell-cell contacts. All of these mechanisms are utilized during the different stages of *Drosophila* development.

Figure 1.3. The different morphogenetic events of *Drosophila* embryogenesis.

Anterior is to the left in all pictures. Red dots mark tricellular junctions. **A.** Surface view of embryonic epidermal cells just after cellularization. The cells adopt a roughly hexagonal shape and each of their vertices contacts three neighbouring cells (red dot). The polarity of the tissue across the dorsal-ventral or the anterior-posterior axis is referred to as planar polarity. **B-H.** Embryos stained for Ed (B–D) or Armadillo (Arm; E-H) to highlight the outlines of the cells. **B.** Sagittal section through a cellularizing embryo. At this stage, the embryo is composed of a single layer of cells. Boxed area is enlarged; at this stage Ed is enriched at the apical portion of the lateral domain. **C.** Surface view of an embryo during ventral furrow invagination. The ventral-most cells constrict their apices before invaginating (arrow). **D.** Lateral view of the surface of an embryo during germ band extension. The lateral epidermis extends posteriorly displacing the caudal end of the embryo dorsally (arrow) until it reaches the posterior limit of the head (arrowhead). **E.** Lateral view of the surface of an embryo during germ band retraction. The caudal end (arrow) returns to its posterior position. The morphology of the amnioserosa cells (asterisk) is noticeably different from that of the lateral epidermis. Boxed area is enlarged to better appreciate the shape of the cells. **F.** Lateral view of the surface of an embryo during the sweeping phase of dorsal closure. Boxed area is enlarged to better display the elongation of the epidermal cells along the dorsal-ventral axis. **G.** Lateral-dorsal view of the surface of an embryo during the zippering phase of dorsal closure. The epidermis closes from both anterior and posterior ends toward the middle. **H.** Dorsal view of the surface of an embryo during the termination phase of dorsal closure. Boxed area displays the disappearing suture where the two sheets of epidermis previously adhered (arrow).

A
B
Apical
Basal
C
D
E
F
G
H

1.5.1 Shaping the *Drosophila* embryo: from monolayer to maggot

Drosophila embryogenesis encompasses a broad range of morphogenetic changes and is divided into 17 stages each characterized by specific developmental processes. During the first stages of embryogenesis, the fertilized zygote undergoes multiple rounds of karyokinesis, nuclear division without cytokinesis, to generate a syncytium of approximately 5000 nuclei sharing one cytoplasm. At the end of the cell cycle 14, the nuclei localize to the periphery of the cell and become encased by plasma membrane in a process called cellularization. During this coordinated event, plasma membrane led by actomyosin rings progressively ingresses and ultimately encloses each nucleus. The surface area of the plasma membrane increases approximately 25 fold to enclose every nucleus thus generating the cells of the blastoderm (Figure 1.3B). The ingrowth of the membrane cannot be explained by the "pulling" of the membrane alone. This increase in plasma membrane requires new membrane to be delivered from the Golgi to the apical, and later lateral, domain of the growing cell (Lecuit and Wieschaus, 2000). As this is the first epidermis created in the life of a fruit fly, cellularization results in the establishment of apical-basal polarity and of the first cell-cell adhesion junctions. The first sign of apical-basal polarity is the apical localization of the polarity protein Baz, which is required for the localization of the synaptotagmin-like protein Bitesize (Harris and Peifer, 2005; Pilot et al., 2006). Bitesize then recruits Moesin, which organizes the cortical actin cytoskeleton into a continuous network at the level of the apical junctional region. Spot adherens junctions visible as punctate enrichment of DE-Cadherin/Arm are initially distributed along the nascent membrane as the actomyosin ring progresses basally (Tepass and Hartenstein, 1994). When the cells are almost fully formed the spot

adherens junctions coalesce to form a discontinuous band of adherens junction near the apical domain, which will resolve into a mature zonula adherens only after germ band extension (Grawe et al., 1996; Müller and Wieschaus, 1996; Tepass, 1996; Tepass and Hartenstein, 1994). After the completion of cellularization, the monolayered blastoderm undergoes a series of morphogenetic changes that shape the embryo and its internal organs (Figure 1.3). Following is a short description of such major events of the life of a fruit fly embryo emphasizing cell movements and shape changes.

Multiple overlapping signals pattern the embryo along the anterior-posterior and dorsal-ventral axes. The establishment of those signals is initiated during oogenesis by the localization of specific mRNAs within the oocyte. The proper integration of the multiple signals is required to generate a head at the anterior, a tail at the posterior and a segmented body in between. The patterning of the epidermis is of greatest importance to understanding its subsequent morphogenesis. The blastoderm embryo is patterned along the dorsal-ventral axis via the Toll/Dorsal pathway, which results in the nuclear accumulation of the Dorsal transcription factor in the ventral-most cells to induce mesoderm fate determination. Dorsal stimulates the transcription of *twist* and *snail*, which act together to establish different cell fates including the mesoderm in the ventral-most cells as well as more lateral cell fates along the dorsal-ventral axis (Jiang et al., 1991). Simultaneously, Decapentaplegic (Dpp) expressed on the dorsal side of the embryo acts as morphogen to induce the amnioserosa and dorsal ectoderm fates (Ip et al., 1991; Raftery and Sutherland, 2003; Ray et al., 1991; Stathopoulos and Levine, 2002). The amnioserosa is an extraembryonic tissue that although will degenerate at the end of embryogenesis, participates actively in multiple morphogenetic processes

(Kiehart et al., 2000). These patterning cues ultimately control the morphogenetic programmes that shape the embryo by specifying the correct cell behaviour in the appropriate groups of cells.

Gastrulation is the first major reorganization of the embryo after cellularization and produces the different germ layers essential to organogenesis. This process involves the internalization of the mesoderm via the invagination of the cells distributed along the ventral midline, and its subsequent separation from the ectoderm. The process of invagination itself involves the tight regulation of the actin cytoskeleton for the coordinated constriction of the apical domain of the ventral cells (Fox and Peifer, 2007; Leptin and Grunewald, 1990; Parks and Wieschaus, 1991). Enrichment of F-actin and myosin II at the cortex coincides with the apical constriction of the presumptive mesoderm. This change in the cytoskeleton is linked to the activation of the Rho1 small GTPase pathway. In summary, the secreted molecule Folded Gastrulation activates the G_{α} protein Concertina, which in turns leads to the activation of the Rho1 activator RhoGEF2 (Fox and Peifer, 2007). Rho1-GTP leads to the activation of Rok, which activates MLC providing the contractile tension essential for the change in the shape of the presumptive mesoderm. Apical constriction of the ventral cells results in the bending and local infolding of the tissue (Figure 1.3C). The internalized cells then undergo epithelial to mesenchymal transition to generate different internal organs of the maggot.

During the process of germ band extension, which occurs in stages 7 to 9, the lateral ectoderm expands along the anterior-posterior axis resulting in an approximate doubling of the length of the germ band (Figure 1.3D). This elongation of the ectodermal tissue along the anterior-posterior axis that takes places in the absence of cellular division is caused mostly by the intercalation of ectodermal cells (Bertet et al., 2004). During this process, cells from dorsal and ventral regions of the ectoderm converge toward the mediolateral midline thus extending the tissue along the anterior-posterior axis. How can epithelial cells attached to one another via cell-cell junctions change position within a tissue? It was found that the cells that intercalate rearrange their adhesion contacts in an ordered directional manner thus progressively changing place with respect to their neighbours. To do so, cells remodel specific contacts by shrinking anterior and posterior contacts irreversibly and then expending their dorsal and ventral contacts (Bertet et al., 2004; Zallen and Wieschaus, 2004). Interestingly, myosin II is enriched at the shrinking anterior and posterior faces of the cells in a planar polarized fashion. In embryos mutant for myosin II heavy chain (MHC; encoded by *zipper*) junction remodeling does not occur and the intercalation process fails (Bertet et al., 2004). Similarly, junction remodeling fails in embryos injected with the Rok inhibitor, Y-27632 suggesting that the accumulation of myosin II results from the activation of the Rho1 pathway (Bertet et al., 2004). The establishment of the planar polarized distribution of myosin II is largely unknown except for the requirement of segment polarity genes that pattern the embryo along the anterior-posterior axis (Zallen and Wieschaus, 2004). Interestingly, Baz also adopts a planar polarized distribution although reciprocal to that of myosin II in the same ectodermal cells (Zallen and

Wieschaus, 2004). Baz is a regulator of epithelial cell polarity required for the proper establishment of adherens junctions and it was thus hypothesized that the planar polarized distribution of Baz assists the remodeling of adherens junctions (Wodarz et al., 2000; Zallen and Wieschaus, 2004). According to this hypothesis, loss of Baz from the shrinking face results in the destabilization of DE-Cadherin adhesion complexes while the enrichment of Baz at the reciprocal face stabilizes the formation of new junctions. However, it is yet unclear whether myosin II and Baz influence the distribution of one another, thus resulting in their reciprocal accumulation, or if their distribution is regulated independently but in parallel via an upstream signal. Interestingly, recent findings in cultured mammalian cells suggest that the activation of Rho1 can affect the distribution of Par-3. Indeed, the activated form of mammalian Rho-kinase phosphorylates Par-3 resulting in the disruption of the Par-3/Par-6/aPKC polarity complex (Sordella and Van Aelst, 2008; Zhang and Macara, 2008). Further investigation is required to determine whether Rho-kinase phosphorylates Baz in *Drosophila* and whether this regulation is sufficient to generate the reciprocal distribution of Baz and myosin II in intercalating cells.

After its full extension, the germ band retracts until the caudal end of the embryo returns to its final posterior position (Figure 1.3E). During this process, which spans stage 12, the amnioserosa becomes progressively exposed on the dorsal side of the embryo. During the retraction of the epidermis, segmental grooves appear along the anterior-posterior axis, the epidermis becomes approximately twice as tall along the dorsal-ventral axis while its length decreases approximately by half. These observations

prompted speculations that germ band retraction was the reverse process of germ band extension and therefore would be caused by cell rearrangements within the epidermis. However, live imaging of embryos expressing GFP markers to highlight the outline of the cells showed that cell intercalation does not participate to the retraction of the germ band (Schöck and Perrimon, 2002a). Instead, in contrast to germ band extension, cell shape changes and tissue movements drive germ band retraction. The amnioserosa cells undergo a remarkable shortening of their dorsal-ventral axis accompanied by an elongation along the anterior-posterior axis. Meanwhile, the first rows of epidermal cells closest to the amnioserosa elongate along the dorsal-ventral axis and shorten slightly along the anterior-posterior axis. Expression of either Rho1-DN or Rho1-CA in the amnioserosa causes severe germ band retraction. However, their expression in the first rows of epidermal cells closest to the amnioserosa has no effect on germ band retraction (Schöck and Perrimon, 2002a). As the Rho1 pathway influences the actin cytoskeleton and cellular adhesion, these results suggest that the regulation of actin-myosin contractility and cellular adhesion in the amnioserosa cells is essential for the retraction of the epidermal tail to the posterior.

1.5.2 Dorsal closure

At the end of germ band retraction, the amnioserosa covers the dorsal side of the embryo and contacts the dorsal edges of the epidermal sheets, which flank the amnioserosa laterally on both sides. The amnioserosa is an extraembryonic tissue that will degenerate at the end of embryogenesis. Dorsal closure, occurs when the two sheets of epidermis migrate dorsally, meet and adhere together at the dorsal midline thus

replacing the amnioserosa and safely wrapping the internal organs of the developing embryo in epidermis (Jacinto et al., 2002). At the end of this process, the seam at the dorsal midline is indistinguishable from the outlines of the other epidermal cells and the segments of the two sheets are perfectly aligned (Figure 1.3H).

Dorsal closure has emerged as a popular model for the study of morphogenesis because of the multiple distinct cellular events involved for the closure of the dorsal epidermis. Moreover, these complex tissue movements require the integration of different signaling pathways to program the different cellular behaviours that participate in dorsal closure. As the amnioserosa eventually degenerates, it was first thought to be passively replaced by the epidermis but it is now known that both the epidermis and the amnioserosa actively participate in dorsal closure (Kiehart et al., 2000). Indeed, the continuous constriction of the amnioserosa cells contributes one of the four known major forces essential to the dorsal migration and the suture of the dorsal epidermis, the other three forces being provided by the epidermis: dorsal-ventral elongation of the epidermal cells, the constriction of an actomyosin cable at the leading edge of the dorsal-most epidermal cells and filopodia and lamellipodia cellular protrusion from the leading edge (Franke et al., 2005; Hutson et al., 2003; Kiehart et al., 2000; Peralta et al., 2007). The entire process of dorsal closure is divided into four phases each characterized by distinct morphological appearance of the epidermis and amnioserosa and during which a different set of forces operates (Jacinto et al., 2002).

1.5.2.1 Initiation: preparing the leading edge

As the germ band retracts, the amnioserosa becomes progressively exposed from anterior to posterior. Dorsal closure begins at the anterior while germ band retraction is nearing an end at the posterior, and subsequently starts at the posterior as well. Initiation spans embryonic stage 12 and during this phase, the leading edge is irregular and scalloped, the dorsal epidermal cells are cuboidal and the actomyosin cable has not formed yet suggesting that the epidermis exerts little tension (Figure 1.3E). It is therefore thought that the net movement of the epidermis during this stage results from the constriction of the amnioserosa thereby tugging the epidermis dorsally (Jacinto et al., 2002).

1.5.2.2 Sweeping: assembly of actomyosin cable and dorsal-ventral elongation of epidermal cells

As initiation proceeds, an actomyosin cable, noticeable by the conspicuous enrichment of F-actin and myosin II, assembles at the leading edge of the dorsal most epidermal cells at their interface with the amnioserosa. At the cellular level, the apparently continuous multicellular cable is actually formed by the polarized accumulation of F-actin and myosin II at the leading edge of each dorsal most epidermal cell. This accumulation of actin and myosin II at the leading edge is one feature of the planar polarity of the dorsal-most epidermal cells, which makes those cells different at the face contacting the amnioserosa from the other faces juxtaposed to epidermal neighbours (Kaltschmidt et al., 2002). This planar polarized accumulation of the actin cytoskeleton suggests that the mechanisms involved in producing an actomyosin cable,

including the Rho1 pathway, are active only at the leading edge and therefore also planar polarized.

The accumulation of F-actin is first detectable at the tricellular junctions of the leading edge of the epidermis (see Figure 1.3F for the position of the actin-nucleating centers) (Kaltschmidt et al., 2002). Those sites, termed (ANCs), were therefore hypothesized to hold the actin polymerization machinery. It was recently found that indeed, Diaphanous (Dia) and Ena accumulate at the ANCs at the time when the actomyosin cable assembles (Gates et al., 2007; Homem and Peifer, 2008). Because the actomyosin cable assembles exclusively at the leading edge of the epidermis, it is tempting to speculate that Rho1 signaling is active only at that face of the cells. However, it is still not clear how the dorsal-most epidermal cells adopt the planar polarity necessary to restrict the activity the actin and myosin II controlling machinery at the leading edge.

The actomyosin cable is under tension; when the cable is cut with a laser, the leading edge cells flanking the wound recoil anteriorly and posteriorly as if pulled by the remaining cable. This response in the cell shape suggests that the entire leading edge is under tension and that the tension generated by each piece of cable within each cell is shared throughout the leading edge possibly via the attachment of the actomyosin cables to intercellular junctions that connect the leading edge cells together (Danjo and Gipson, 1998). The appearance of the actomyosin cable coincides with the smoothening of the leading edge, suggesting that the actomyosin cable "pulls" the leading edge cells

into a taut interface. This taut interface describes an eye shape amnioserosa domain on the dorsal side (Figure 1.3F).

1.5.2.3 Zippering: knitting together two epidermal sheets

As the anterior and posterior corners (canthi) of the advancing epidermal sheets come into close contact, filopodia and lamellipodia extensions reach across over the amnioserosa and zip the epidermis together. The eye-shaped opening thus progressively zips from the anterior and posterior canthi toward the middle (Figure 1.3G). The zippering of opposing epidermal cells occurs with remarkable accuracy, with the patterning of the epidermal segments perfectly maintained across the midline seam at single-cell resolution. In order to achieve this level of accuracy, each cell in the leading edge must be able to identify and adhere with its matching partner on the opposing epithelial sheet. This robust matching process involves a yet unclear mechanism to perfectly align the segments on the dorsal side of the embryo. What is known is that filopodia extending from contralateral epidermal cells are crucial for the epidermal cells to "sample" the cells before matching and adhering. This hypothesis was prompted after the observation that mutations that abolish filopodia, such as expression of Cdc42-DN, the sequestration of the actin regulator Ena or the disruption of microtubules, all result in the loss of filopodia and subsequent mismatching of segments during zippering (Gates et al., 2007; Jacinto et al., 2000; Jankovics and Brunner, 2006). But what could the filopodia be sensing to recognize their binding partner? It was recently found that the matching of the epidermal cells is linked to their anterior-posterior segmental patterning, which occurs early during embryogenesis. Early in development, the

embryo is divided into a series of repeating units called parasegments, by the integration of different signaling pathways (Lawrence and Struhl, 1996). Later in embryogenesis, visible segment boundaries form and the embryo is patterned with each segment being divided into an anterior and a posterior compartment by the parasegment boundary (Larsen et al., 2003). To determine whether the filopodia from cells belonging to different segmental compartments interact together during the zippering phase, stripes of cells belonging to different compartments were marked with different colored fluorescent tags (Millard and Martin, 2008). The actin-binding domain of Moesin was tagged with either a green fluorescent protein (GFP) or a red fluorescent protein (RFP) and both constructs were simultaneously expressed one in the anterior and the other in the posterior compartment of each segment. Using this tool, Millard and Martin found that filopodia extending from the same compartment on either side of the amnioserosa can recognize and bind to each other whereas filopodia of cells belonging to different compartment do not persistently interact. Although the matching is specific to the compartment, it is not specific to the segment. Indeed, if the mismatch is great enough that filopodia can reach the equivalent compartment of a neighbouring segment, the filopodia can bind resulting in the adhesion of the leading edge cells and mismatching of the segments at the midline.

Once the filopodia have made contact, they adhere to each other and drag the epidermis shut at the dorsal midline (Jacinto et al., 2002). Continuous ablation of the filopodia at the canthi prevents closure from the anterior and posterior toward the middle (Peralta et al., 2007). However, the sustained constriction of the amnioserosa is

sufficient for bringing the opposing leading edges together thus allowing ectopic zippering internally, between the two endogenous canthi.

1.5.2.4 Termination: blending in at the seam

In this final step of dorsal closure, the two adjacent epidermal sheets stop advancing and seal at the dorsal midline into a continuous seamless tissue (Figure 1.3H). This process, although still quite nebulous, most likely involves some type of contact inhibition that deactivates the different forces of morphogenesis, promotes the disassembly of the actomyosin cable and allows the detachment of the epidermal cells from the dying amnioserosa cells. As mentioned previously, as opposing cells approach, their leading edges extend filopodia and lamellipodia, which participate in both, the recognition of matching leading edge cells in the opposing sheet and the tugging of the epidermis together (Jacinto et al., 2002). How do the zipping leading edges mature to form an invisible seam at the dorsal midline? While dorsal closure termination has not been as extensively investigated as the other phases of dorsal closure, some work performed on mammalian cell culture provides interesting clues as to how the initial puncta of adhesion mature into robust adherens junctions.

Cultured cells grown to confluence in a calcium-poor medium initiate the formation of adherens junctions upon addition of calcium-rich medium. This malleable system provides a powerful model for the study of adherens junction formation. Using this system, Vasioukhin and colleagues studied the mechanisms that provide the force necessary to actively bring primary keratinocytes, a type of epithelial cells, together and

seal them into a continuous sheet (Vasioukhin et al., 2000). They discovered that when stimulated by addition of calcium, primary keratinocytes extend filopodia that interdigitate upon contact with neighbouring cells. Interestingly, this interlocked array of filopodia forms an adhesion zipper characterized by foci containing E-cadherin and actin interacting proteins including the mouse homolog of Ena, Mena/VASP. Robust adherens junctions progressively replace these adhesion zippers by the rearrangement of the underlying cortical actin cytoskeleton. This process is reminiscent of the zippering of the *Drosophila* epidermis during dorsal closure. Indeed, Ena accumulates at tricellular junctions along the leading edge during dorsal closure and is essential for the proper zippering (Gates et al., 2007). Because of the similarities between the adhesion zipper mechanism of primary keratinocytes and the adhesion of the lateral epidermis during dorsal closure, these observations made on primary keratinocytes may represent a mechanism also applicable to the maturation of the dorsal seam during termination of dorsal closure.

1.5.3 Patterning the dorsal epidermis, leading edge and amnioserosa

The behaviour of cells and tissues during morphogenesis is pre-programmed in the specification of the fate they previously adopted during their development. The different cell types implicated in dorsal closure, the dorsal epidermis and amnioserosa, are fated early during embryogenesis at the blastoderm stage. At that stage, an interplay of signals pattern the embryonic monolayer along the dorsal-ventral axis. The secreted molecule Dpp acts as a morphogen and induces different cell types on the dorsal side of the embryo according to the concentration the cells perceive (Ip et al., 1991; Raftery and

Sutherland, 2003; Ray et al., 1991; Stathopoulos and Levine, 2002). The dorsal-most cells of the blastoderm receive the highest level of Dpp signal become the amnioserosa cells. Just lateral to the amnioserosa, the next rows of cells receive moderate levels of Dpp and adopt the dorsal ectodermal cell fate. This initial fate map establishes the future morphological programmes of these two tissues during dorsal closure.

The dorsal-most epidermal cells that contact the amnioserosa, the leading edge cells, exhibit particular behaviours and cell shape changes during dorsal closure and their fate is regulated by more than the initial Dpp signal that occurs in the blastoderm. Indeed, dorsalizing mutations that expand the domains of amnioserosa and dorsal epidermis fates display no expansion of the leading edge cell fate (Stronach and Perrimon, 2001). Rather, the first row of dorsal epidermal cells that contacts the amnioserosa invariably adopts the leading edge fate, suggesting that it is the juxtaposition of those two cell types that triggers the leading edge cell fate. Some of the initial work on dorsal closure revealed a central role for the Jun amino-terminal kinase (JNK) signaling cascade, a member of the family of Mitogen-Activated Protein Kinase (MAPK) (Glise and Noselli, 1997; Harden, 2002; Jacinto et al., 2002; Martin-Blanco, 1997; Noselli and Agnes, 1999; Xia and Karin, 2004). The JNK cascade, like other MAPK cascades, involves different kinases that act in a hierarchal manner by activating their target through phosphorylation. JNK (encoded by *basket*) is phosphorylated by JNK kinase (JNKK; encoded by *hemipterous*), which in turn is activated by JNKK kinase (JNKKK; encoded by *slipper*). The result of the activation of JNK is the phosphorylation and nuclear translocation of the AP-1 transcription factor composed of

Drosophila Jun (DJun) and *Drosophila* Fos (DFos; encoded by *kayak*). Once in the nucleus, the AP-1 transcription factor regulates the expression of several genes including *dpp* and *puckered* (*puc*) (Glise and Noselli, 1997; Ricos et al., 1999; Riesgo-Escovar and Hafen, 1997). During dorsal closure, the leading edge exhibits high levels of JNK activity, visualized by the expression of the enhancer traps *dpp-lacZ* or *puc-lacZ* or again by the nuclear localization of either DJun or DFos. The protein phosphatase Puc limits the activity of JNK by de-phosphorylating it thus keeping a proper balance of JNK activity. This pathway is essential for the elongation of the epidermal cells along their dorsal-ventral axis during dorsal closure and any mutation that alters the balance of JNK activity either by increasing (*puc* mutation) or decreasing (*basket* mutant) JNK activity severely disrupts dorsal closure (Martin-Blanco et al., 1998). The dorsal closure phenotypes of different mutants in the JNK pathway all share common characteristics: failure in the dorsal-ventral elongation of the epidermis, lack of the actomyosin cable and filopodia from the leading edge, and finally the presence of a gaping hole on the dorsal side of the embryo at the end of embryogenesis.

While JNK activity is essential in the epidermal cells, JNK activity must be turned off in the amnioserosa (Reed et al., 2001). During germ band retraction, the amnioserosa cells and the abutting epidermal cells express high levels of JNK activity. However, prior to the sweeping phase of dorsal closure, JNK activity is turned down specifically in the amnioserosa by the double action of the transcription factor Hindsight and the phosphatase Puc. When JNK activity remains high in the amnioserosa (*hindsight* or *puc* mutants), dorsal closure fails and reduction of JNK activity in

hindsight mutant embryos rescues the dorsal closure defects indicating that high levels of JNK in the amnioserosa are deleterious to dorsal closure.

The JNK pathway influences the actin cytoskeleton as embryos mutant for the JNK pathway exhibit aberrant accumulation of F-actin and myosin II and few or no filopodia are at their leading edge. In a microarray analysis to identify downstream targets of JNK, it was found that JNK regulates the expression of adhesion molecules and actin regulators, including Profilin (Jasper et al., 2001). Interestingly, embryos that lack Profilin (*chickadee* mutants) fail to assemble an actomyosin cable and filopodia at the leading edge, and display a dorsal hole phenotype similarly to JNK pathway mutant embryos (Jasper et al., 2001).

1.5.4 Rho small GTPases during dorsal closure

The Rho family of small GTPases is instrumental to the signaling pathway and cytoskeletal changes that drive dorsal closure. Five members of the Rho small GTPases are known to affect dorsal closure: three Racs (Rac1, Rac2 and Mtl), Rho1 (also called RhoA) and Cdc42 (Johndrow et al., 2004). Most of our knowledge about the function of the Rho small GTPases in dorsal closure comes from the expression of dominant negative and constitutively active alleles. Moreover, the recent isolation of mutations in the different Rho small GTPases has brought further information about their participation to morphogenesis in *Drosophila*.

As expected from the known function of Rho1 in cultured cells, Rho1 is essential for the assembly of the actomyosin contractile cable in the epidermal cells. Expression of Rho1-DN in the epidermal cells results in the loss of the actomyosin cable and embryos mutant for *rho1* display a similar defect in the assembly of the actomyosin cable (Bloor and Kiehart, 2002; Magie et al., 1999). Cdc42 is involved in the formation of the filopodia and therefore for the proper alignment of the epidermal segments during the zippering phase. Expression of Cdc42-DN in the epidermis results in the absence of filopodia (Jacinto et al., 2000). Unfortunately, *cdc42* mutant embryos die during germ band retraction, before dorsal closure, and therefore the results of the dominant negative Cdc42 construct expression have not been confirmed yet (Genova et al., 2000). The expression of Rac1-DN in epidermal cells results in the absence of the actomyosin cable and filopodia in the leading edge cells while the expression of Rac-CA results in strong accumulation of F-actin and numerous protrusions from the leading edge (Harden et al., 1995; Woolner et al., 2005). Similarly, embryos mutant simultaneously for all three Racs, *rac1*, *rac2* and *mtl* also display defects in the actin cytoskeleton similar to that of the dominant negative Rac expressing embryos (Wood et al., 2002). Additionally, embryos that express a dominant negative allele of *rac* in the epidermis lack JNK activity while the expression of a constitutively active form of Rac in the epidermis results in increased JNK activity (Glise and Noselli, 1997). Therefore, the activity of Rac1 modulates the actin cytoskeleton in the leading edge perhaps via the regulation of the JNK pathway. Unlike the Racs, Rho1 has no effect on JNK activity (Magie et al., 1999). However, the expression of Cdc42-DN results in a decrease in Dpp expression in the cells of the leading edge (Glise and Noselli, 1997). This effect is not seen in

cdc42 mutant embryos, which do not exhibit a loss in Dpp, suggesting that the phenotype seen with Cdc42-DN might result from non-specific activity of the transgene (Genova et al., 2000).

1.6 *Drosophila* oogenesis as model for tissue remodeling

Each egg laid by a fruit fly is the product of the development of a unit called egg chamber, each of which is composed of a cyst of 16 interconnected germline cells surrounded by a monolayered epithelium of somatic follicle cells. As the major axes of the future embryo are established in the developing egg chamber, *Drosophila* oogenesis has been the focus of many genetic analyses to elucidate the complex interplay among the different signaling pathways that pattern the germline. However, the follicular epithelium exhibits a diverse range of morphological changes throughout oogenesis making it a fascinating model for the study of tissue rearrangements.

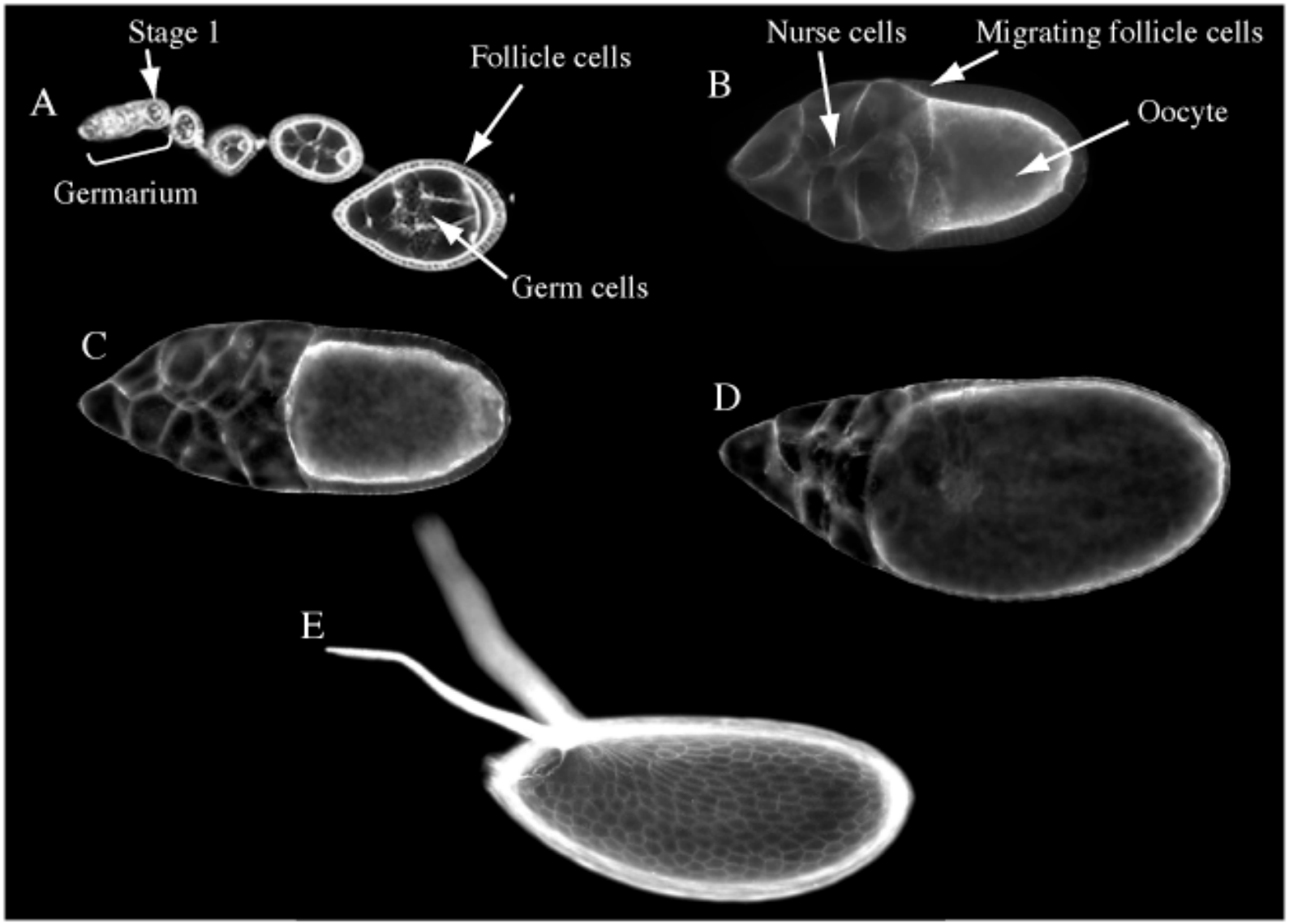
Drosophila oogenesis is divided into 14 developmental stages, each of which is defined by particular morphological features of the egg chamber with the 14th stage being the mature egg ready to be fertilized and deposited (Figure 1.4). The first step in the formation of an egg chamber takes place in the germarium, the region that houses the germline and follicle cell stem cells, when a germline stem cell divides asymmetrically to generate a cystoblast (Figure 1.4A). The cystoblast then undergoes four consecutive rounds of mitotic divisions with incomplete cytokinesis producing a cyst of 16 germline cells interconnected by actin-rich ring canals, which are remnants of the incomplete cytokinesis. One of the 16 germline cells becomes the oocyte while the

others become the polyploid nurse cells that support the growth of the oocyte (Horne-Badovinac and Bilder, 2005).

The completion of the 16-cell cyst is coordinated with its encapsulation by approximately 80 follicle cells, producing a spherical stage 1-egg chamber. The nascent egg chamber then buds from the germarium and begins its development, a journey through which the egg chamber will progressively morph into a mature egg. Throughout oogenesis, the egg chamber will dramatically change in shape mostly due to the morphogenesis of the follicular epithelium. The follicle cells have multiple roles during oogenesis: including the conveyance of patterning cues to the underlying germline, the production and deposition of yolk for the oocyte, and the secretion of the eggshell. The establishment of proper apico-basal polarity of the follicular epithelium is fundamental to the accomplishment of those tasks (Spradling, 1993).

Figure 1.4. ***Drosophila*** **oogenesis.**

A-D. Optical sagittal cross sections of *Drosophila* egg chambers stained with fluorescent phalloidin to highlight the outlines of the cells. **A.** String of early stage egg chambers tipped at the anterior with the germarium. During those early stages, the follicle cells surround the germline cells as uniform cuboidal epithelium. **B.** Stage 9 egg chamber displays the posterior migration of most of the follicle cells (short arrows) toward the oocyte. The few follicle cells that remain associated with the nurse cells spread very thin and cannot be seen in this image. **C.** Stage 10 egg chamber; most of the follicle cells lie on top of the oocyte as a columnar epithelium. **D.** Stage 11 egg chamber; the follicle cells stretch to accommodate the growing volume of the oocyte thus becoming a squamous epithelium. **E.** Surface view of an egg. The eggshell is patterned by the imprints of the follicle cells that secrete the eggshell. The two dorsal appendages extend anteriorly. Anterior is to the left in all pictures.


Stage 1
Follicle cells
A
Germarium
Germ cells
Nurse cells
Migrating follicle cells
B
Oocyte
C
D
E

1.6.1 Apico-basal polarization of the follicular epithelium

All epithelia exhibit a characteristic apico-basal polarity that compartmentalizes their plasma membrane and underlying cytoskeleton into functional regions. The apical domain of the follicular epithelium contacts the germline while the basolateral domain contacts neighbouring follicle cells and the basement membrane. Different protein complexes distinguish the apical and basolateral domains of the plasma membrane. The apical domain, comprised of the free apex and the marginal zone, exhibits the presence of Crumbs (Crb), a single-pass transmembrane protein, and its binding partners Patj and Stardust (Std) (Müller, 2000; Tepass, 2002; Tepass et al., 2001). Crumbs is the only known protein that has the ability to promote apicalization in epithelial cells; loss of Crumbs results in the loss of the apical domain while its overexpression causes an expansion of the apical domain of epithelial cells (Wodarz et al., 1995). The adherens junction belt lies immediately basal to the marginal zone on the lateral domain of the cells and is characterized by the enrichment of the DE-Cadherin/Arm complex and cortical actin cytoskeleton. Basal to the adherens junctions on the lateral side of the cells lie the septate junctions, an invertebrate specific adhesion site populated by adhesion proteins such as Fasciclin III (Fas III), Coracle (Cor) and the protein complex Lethal Giant larvae, Disc Large and Scribble (Lgl, Dlg, Scrib). The basal domain is enriched with Integrin adhesion molecules and their binding partners, which together mediate and regulate cell-matrix adhesion with the basement membrane.

The polarization of the follicle cells along their apicobasal axis is established as the follicle cell stem cells divide. The contact with the basement membrane is the first polarization signal and establishes the basal domain by the localization of integrin mediated cell-matrix adhesion. Additionally, upon contact between the follicle cells and the germline cells, Crb localizes at the apical membrane along with its binding partners PatJ and Std. Contact with the germline is crucial for apical polarization; agametic ovaries produce follicle cells that exhibit lateral and basal polarity but lack apical identity (Tanentzapf et al., 2000). Interestingly however, the basolateral domain of follicle cells is properly established in such ovaries.

1.6.2 The different migrations of the follicular epithelium

The 14 developmental stages of oogenesis refer to morphological characteristics of the egg chamber, which differ greatly between the nascent stage 1-egg chamber that buds from the germarium to the mature stage 14-egg. The most obvious differences among the stages are the shape and size of the egg chamber. A stage 1-egg chamber is a sphere of approximately 30 μm in diameter, which through development becomes an oval egg of approximately 520 μm in length at stage 14 (Horne-Badovinac and Bilder, 2005; Spradling, 1993). To accommodate this dramatic increase in size, the initial 80 follicle cells divide to increase their number to about 650 by stage 6 at which time, their division stops. From stage 1 to stage 8, the follicle cells form a uniform epithelium of cuboidal cells surrounding the growing germline. The transition from stage 8 to 9 is highlighted by the most striking changes in the follicular epithelium: the concerted migration of about 600 follicle cells toward the posterior to cover the oocyte, the

delamination and posterior migration of the border cells in between the nurse cell cluster to reach the anterior border of the oocyte and the dramatic stretching of approximately 50 anterior follicle cells that remain to cover the nurse cells (Horne-Badovinac and Bilder, 2005). For the purpose of this work, I will focus on the posterior migration of the follicular epithelium.

1.6.2.1 Stage 9: Extreme makeover

The transition from a stage 8- to a stage 10-egg chamber is highlighted by remarkable changes in the morphology of the follicle cells. Over a period of about 6 hours, 600 posterior follicle cells change their shape from cuboidal to columnar and align precisely to cover the oocyte. While the posterior follicle cells change their shape and pack themselves tightly together over the oocyte, the remaining approximately 50 anterior follicle cells stretch thin to cover the nurse cells (Figure 1.4B,C) (Horne-Badovinac and Bilder, 2005; Spradling, 1993).

During this stage, the follicle cells migrate *en masse* toward the oocyte by changing their substratum from nurse cells to oocyte. During this movement, follicle cells maintain contact with the same neighbours. Although these changes in morphology are striking, the molecular signals that control them are still disappointingly uncharacterized. For example, it is unknown what molecular changes create the abrupt boundary between the nurse cell associated stretched follicle cells and the oocyte associated columnar follicle cells.

Even with little molecular knowledge of this process, it seems likely that the remodeling of the actin cytoskeleton and the regulation of both cell-cell and cell-matrix adhesion are essential for the posterior migration of the follicle cells. In addition, it is tempting to speculate that differential adhesive properties establish the dramatic boundary between the stretched and columnar follicle cells, while continuous adhesion is required for the cohesion between the last stretched cell and the first columnar cell to maintain the integrity of the epithelium.

1.6.3 Appendage tube formation

Late during oogenesis, the follicle cells secrete the eggshell material through their apical domain onto the underlying oocyte. The *Drosophila* eggshell is decorated by two respiratory appendages at the dorsal anterior that protrude out of the plane of the eggshell. How can a flat sheet of epithelial cells secrete those dorsal appendages? Late during oogenesis, specialized groups of follicle cells, termed appendage primordia, re-organize from a flat sheet into tubes and it is the secretion of eggshell material inside those tubes that generates the dorsal appendages (Dorman et al., 2004). To understand the morphogenetic events that result in the formation of the appendages, we must first look at the signals that pattern the appendage primordia.

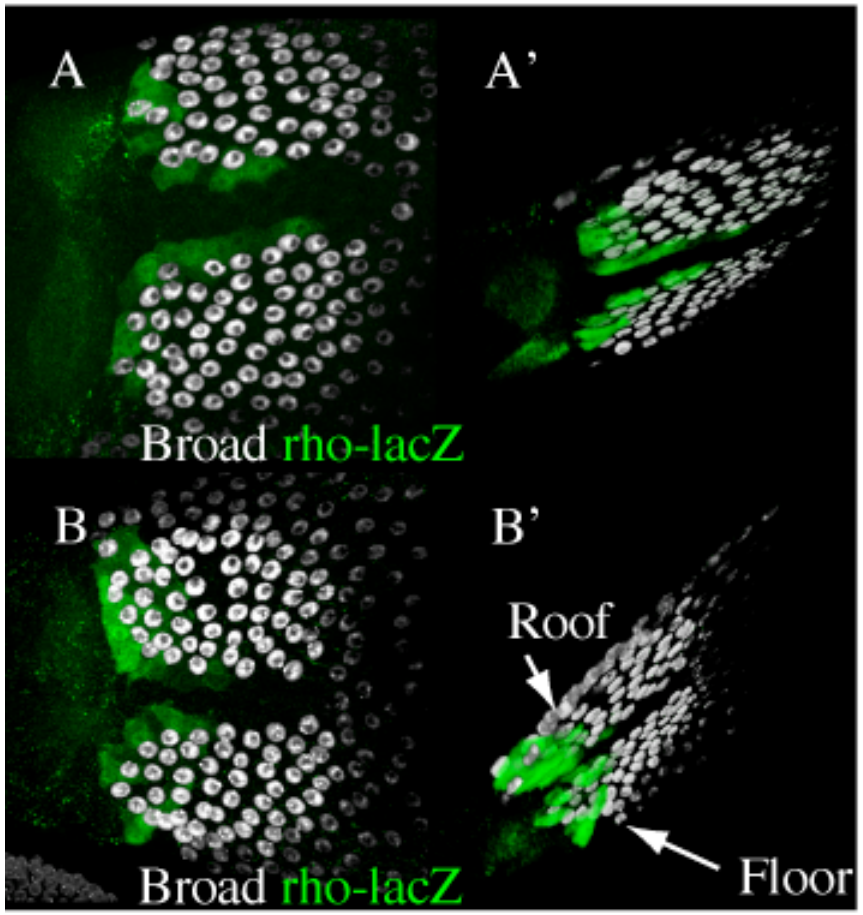
The complex and intrinsic combination of signaling cascades that pattern the egg chamber has been the subject of multiple studies over the past decades. Gurken (Grk), a homolog of the Transforming Growth Factor alpha is produced by the oocyte and secreted into the space between the oocyte and the follicular epithelium where it acts as

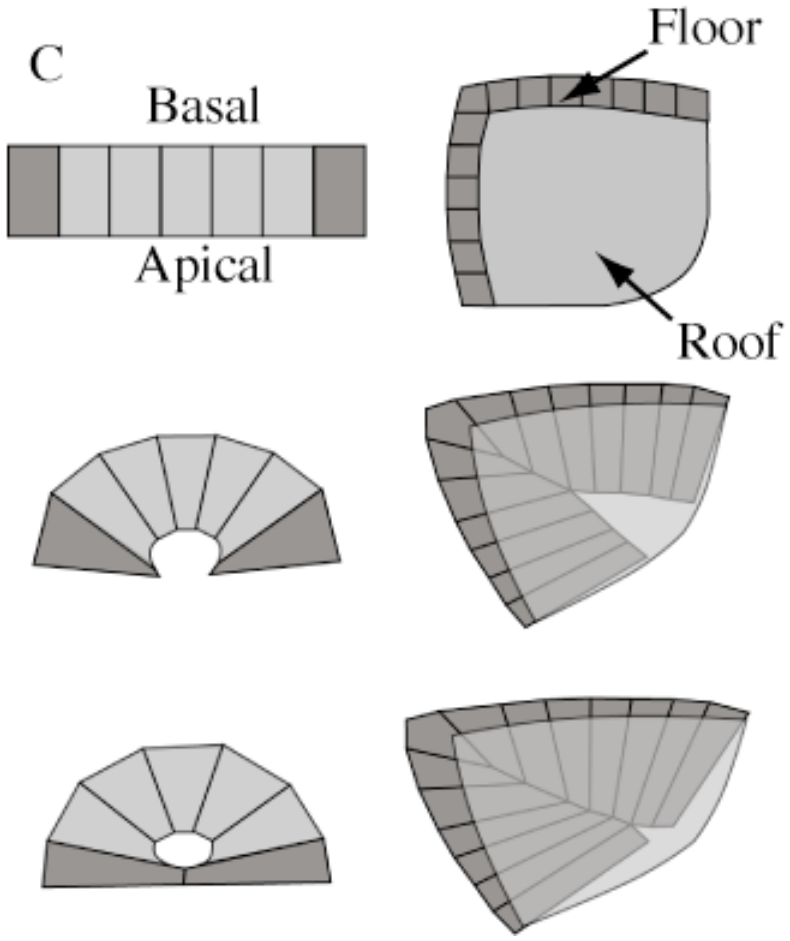
ligand for the Epidermal Growth Factor Receptor (Egfr) expressed by all the follicle cells (Nilson and Schupbach, 1999). As Grk concentrates in a crescent at the dorsal anterior of the oocyte just above the oocyte nucleus, only the follicle cells of the dorsal anterior will receive this signal. The Grk/Egfr pathway patterns a field of cells at the dorsal-anterior and the interplay between the downstream effectors of this pathway refines the dorsal field into populations of cells of different fate distinguishable by the expression of different markers.

One such marker is the transcription factor Broad Complex (thereafter referred to as Broad), which is initially expressed at moderate levels in all the follicle cells. Upon Grk/Egfr signaling, the expression of Broad is turned off in the dorsal midline while it is increased in two populations of follicle cells on either side of the midline (Deng and Bownes, 1997). Each patch of high Broad expressing cells comprises one of the two cell types that populate an appendage primordium. The other cell type flanks the high Broad expressing cells as a single row of cells organized in an L-shape at the dorsal and anterior borders and is marked by the expression of the promoter construct *rhomboid-lacZ* (Ward and Berg, 2005). These two cell types will reorganize into appendage secreting tubes and at the end of this morphogenesis, the high Broad expressing cells, termed roof cells, will populate the roof of the tubes while the *rhomboid-lacZ* expressing cells, the floor cells, will populate the floor of the tube (Figure 1.5A-B').

Figure 1.5. The dorsal appendage secreting tubes are shaped by the coordinated movements of roof and floor cells.

Egg chambers stained for the roof cell marker Broad (BrC; white nuclear staining) and the floor cell marker rho-lacZ (green cytoplasmic staining). **A.** Projection of multiple focal planes taken along the z-axis of a stage 11 egg chamber. The floor cells arranged in a hinge flank the roof cells at the anterior and medial axes. The floor cells have started to elongate underneath the roof cells (arrow points to the extending tip of the floor cells). **A'.** Rotated three-dimensional reconstruction of A shows the floor cells underneath the roof cells. **B.** Projection of multiple focal planes taken along the z-axis of a stage 12 egg chamber. At this later stage, the floor cells have extended further underneath the roof cells (arrow points to the extending tip of the floor cells). **B'.** Rotated three-dimensional reconstruction of A shows the floor cells underneath the roof cells. Anterior is to the left in all pictures. **C.** Schematic diagram of the cell shape changes that occur during appendage tube closure. Cross-section (left) and surface view (right). The floor cells are in dark grey and roof cells are in pale grey.


A
A'
Broad rho-lacZ
B
B'
Roof
Broad rho-lacZ
Floor


C
Basal
Apical
Floor
Roof

The roof and floor cells are distinguishable from the other follicular cells by the specific markers they express but also by their particular shape. While the other oocyte associated follicle cells flatten from columnar to squamous as the oocyte grows in volume, the cells of the appendage primordia remain columnar resulting in an apparent thickening of the epithelium in the dorsal-anterior region (Dorman et al., 2004). Their reorganization into a tube involves specific cell shape changes particular to each roof and floor cell fate (Figure 1.5C). The roof cells constrict their apices thereby bending the epithelium upward while the floor cells elongate underneath the elevated roof cells (Figure 1.5C). The constriction of the roof cells is accompanied by an enrichment of F-actin at their apical domain as well as an enrichment of the actin regulator Ena (C.L. unpublished observations). The high expression of Broad is essential and sufficient for the apical constriction of the roof cells: clones of *broad* mutant roof cells fail to constrict while clones of follicle cells that ectopically overexpress Broad constrict their apices (Atkey et al., 2006; Ward et al., 2006). As the floor cells elongate, their apical domains draw nearer until they meet and bind (Figure 1.5C). The binding of the floor cells together closes the floor of the appendage tube. A contractile actomyosin cable assembles at the interface of the floor and roof cells and participates in the closure of the floor cells together: removal of this actomyosin cable prevents the closure of the floor cells and result in an open floor phenotype (Laplante and Nilson, 2006). The closed tube will then extend anteriorly by the migration of the floor cells over the nurse cells. This elongation event occurs as the floor cells migrate over the nurse cells. Finally, secretion of eggshell material into these tubes molds the final shape of the appendages.

1.7 Differential expression of Echinoid influences the actin cytoskeleton

1.7.1 Echinoid

We still have much work to do before we understand how cells regulate their cytoskeleton to drive cell shape changes and morphogenesis. During my Ph.D. work, I have focused on the role of the cell adhesion molecule Echinoid (Ed) in the formation of contractile actomyosin cables during different morphogenetic events of *Drosophila* development. Ed is an Immunoglobulin domain containing adhesion molecule that interacts homophilically in *trans* (Laplante and Nilson, 2006; Wei et al., 2005) and heterotypically with Neuroglian (Islam et al., 2003). It was fist studied for its role in modulating cell-cell signaling by Notch and the Epidermal Growth Factor Receptor (EGFR). Ed interacts genetically with the Notch signaling pathway to enhance the lateral inhibition role of Notch (Ahmed et al., 2003; Escudero et al., 2003). Also, Ed antagonizes EGFR signaling during photoreceptor fate determination in the *Drosophila* eye (Bai et al., 2001; Rawlins et al., 2003; Spencer and Cagan, 2003).

1.7.2 Differential expression of Ed triggers the assembly of actomyosin cables

The isolation and characterization of a novel allele of *echinoid* (*ed*), named ed^{F72}, is at the source of my passion for the actin cytoskeleton and its regulation. The ed^{F72} allele was isolated in a mutagenesis screen to identify genes with mosaic eggshell defects. Female *Drosophila* mosaic for ed^{F72} lay eggs that exhibit a pattern of eggshell

imprints surrounded by smooth boundaries. This is in contrast with clones of wild type cells, which exhibit a jagged interface with their neighbours at clone borders. As the eggshell imprints represent the organization of the follicle cells that secreted them, I studied the organization of the follicle cells in ed^{F72} mosaic follicular epithelia. Through that work, I found that the interface between Ed expressing and non-expressing follicle cells was smooth, similar to the eggshell imprints. Further investigation of this phenotype showed that the juxtaposition of Ed expressing and Ed non-expressing follicle cells triggers the assembly of a contractile actomyosin cable at clone interfaces marked by the enrichment of F-actin and the active phosphorylated form of MLC (Laplante and Nilson, 2006). Moreover, adherens junctions at the border of ed^{F72} are sometimes lost or appear discontinuous indicating that the differential expression of Ed between two tissues can affect the stability of adherens junctions. For example, the destabilization of the adherens junctions could affect the actin cytoskeleton and trigger the formation of an actomyosin cable or the formation of the actomyosin cable could affect the stability of the adherens junctions. These phenotypes were also observed in *ed* mutant imaginal wing disc cell clones (Lecuit, 2005; Wei et al., 2005).

The expression of Ed is very dynamic during both *Drosophila* oogenesis and embryogenesis. In the ovary, this interface lies between the two cell types of the dorsal appendage primordia. In the embryo, the absence of Ed from the amnioserosa during dorsal closure generates an Ed expression interface with the lateral epidermis, which coincides with the well-characterized actomyosin cable present in the epidermal cells at this interface. In both cases, elimination of Ed leads to the loss of the actomyosin cable

causing subsequent defects in morphogenesis (Laplante and Nilson, 2006). This work prompted the hypothesis that the differential expression of Ed between two neighbouring tissues results in the formation of an actomyosin cable at their interface.

1.7.3 The molecular function of Echinoid

Little is known about the molecular function of Ed. Recent work has focused mainly on the only known motif in the cytoplasmic tail of Ed, a PDZ domain binding motif located at the very carboxy-terminus, which acts as an anchor for PDZ domain containing proteins. The combined effort of different studies has proposed multiple PDZ containing partners for Ed: Baz/PAR-3, Canoe (Cno; the *Drosophila* homologue of Afadin), Jaguar/MyosinVI and the *Drosophila* Glutamate Receptor Interacting Protein (DGrip) (Lin et al., 2007; Swan et al., 2006; Wei et al., 2005). These different interaction partners suggest that Ed is either a very promiscuous protein in its interactions or perhaps that each different interaction is specific to a particular function of Ed at a certain time and in a certain tissue.

Most relevant to this work are the interactions between Ed and both the actin binding protein Cno and the polarity protein Baz. It was shown through *in vitro* binding assays and co-immunoprecipitation of whole tissues that Ed can interact with either Baz or Cno and that the PDZ domain binding motif of Ed is required for these interactions (Wei et al., 2005). As Baz can interact with either Ed or Armadillo, which bind DE-cadherin, it was concluded that Ed cooperates with DE-Cadherin in cell adhesion and that they redundantly position Baz to adherens junctions. The intimate ties between

adherens junction stability and the regulation of the actin cytoskeleton thus prompted the hypothesis that the role of Ed as a modulator of adherens junction stability can influence the formation of actomyosin cables.

Chapter 2: Differential expression of the adhesion molecule Echinoid drives epithelial morphogenesis in *Drosophila*

2.1 Abstract

Epithelial morphogenesis requires cell movements and cell shape changes coordinated by the modulation of the actin cytoskeleton. We identify a role for Echinoid (Ed), an immunoglobulin domain-containing cell adhesion molecule, in the generation of a contractile actomyosin cable required for epithelial morphogenesis in both the *Drosophila* ovarian follicular epithelium and embryo. Analysis of *ed* mutant follicle cell clones indicates that the juxtaposition of wild type and *ed* mutant cells is sufficient to trigger actomyosin cable formation. Moreover, in wild type ovaries and embryos, specific epithelial domains lack detectable Ed, thus creating endogenous interfaces between cells with and without Ed; these interfaces display the same contractile characteristics as the ectopic Ed expression borders generated by *ed* mutant clones. In the ovary, such an interface lies between the two cell types of the dorsal appendage primordia. In the embryo, the absence of Ed from the amnioserosa during dorsal closure generates an Ed expression border with the lateral epidermis, which coincides with the actomyosin cable present at this interface. In both cases, *ed* mutant epithelia exhibit loss of this contractile structure and subsequent defects in morphogenesis. We propose that local modulation of the cytoskeleton at Ed expression borders may represent a general mechanism for promoting epithelial morphogenesis.

2.2 Introduction

The morphogenesis of diverse epithelia during development derives from coordinated remodeling of cell shape and intercellular interactions that drives cell movements and reorganization (Pilot and Lecuit, 2005)(Schock and Perrimon, 2002b). These changes typically arise from modulation of the actin cytoskeleton. Distinct cell behaviors are associated with specific cell types, but how the determination of cell fate engages the subcellular mechanisms that drive morphogenesis is not well understood. We have used the *Drosophila* ovary and embryo as model systems to study how interactions between cell types lead to local changes in the cytoskeleton that mediate epithelial morphogenesis.

In the ovary, the follicular epithelium surrounds individual cysts of germline cells, each of which gives rise to a single egg (Spradling, 1993). Late in oogenesis, the follicle cells secrete the eggshell, which exhibits pronounced asymmetries produced by specialized follicle cell domains (Berg, 2005) (Dobens and Raftery, 2000). The most prominent features are the two appendages that project from the dorsal anterior region of the eggshell (Figure 2.1A). The follicle cell primordia that produce these appendages are specified in midoogenesis and flank the dorsal anterior midline of the epithelium. Subsequently, the two cell types that comprise the primordium undergo a series of coordinated cell shape changes that remodel the flat primordia into epithelial tubes that then extend anteriorly. Secretion of chorion into the lumen of each tube produces the appendages (Dorman et al., 2004; Ward and Berg, 2005). The signals that specify the

fate and position of the appendage primordia are well-understood (Berg, 2005; Nilson and Schupbach, 1999; Roth, 2003), but the changes in cell shape and organization that occur during the morphogenesis of these primordia have only recently been described in detail (Dorman et al., 2004; Ward and Berg, 2005). Mutations that disrupt appendage morphology downstream of primordia specification have been identified, and thus may affect factors that contribute specifically to tube formation (Berg, 2005), but the molecular mechanisms that control these morphogenetic movements remain unknown.

In contrast, the cytoskeletal forces driving epithelial movements during *Drosophila* embryonic dorsal closure have been well characterized. Dorsal closure occurs when two lateral epidermal sheets move dorsally over the extraembryonic amnioserosa and converge at the dorsal midline, sealing the dorsal side of the embryo. These movements are driven by multiple forces, including tissue-specific changes in the shape of individual cells as well as the tension generated by a supracellular contractile actin cable that arises at the interface of the lateral epidermis and amnioserosa (Kiehart et al., 2000)(Jacinto et al., 2002; Martin and Parkhurst, 2004). Differential activity of the Jun N-terminal kinase pathway between the epidermis and amnioserosa is involved in generating these forces (Reed et al., 2001), but how this difference in activity produces a local effect on the cytoskeleton at the interface between these tissues is not understood.

We demonstrate that Echinoid (Ed), a cell adhesion molecule and adherens junction component (Bai et al., 2001; Islam et al., 2003; Spencer and Cagan, 2003; Wei

et al., 2005) is required for the epithelial sheet movements that occur during appendage primordia morphogenesis and dorsal closure. Specifically, we find that the juxtaposition of cells expressing and lacking Ed induces the assembly of a contractile actomyosin cable at their interface. We initially identified a mutant allele of *ed* based on the smooth borders exhibited by homozygous mutant follicle cell clones, and found that the apical clone border displays morphological and molecular characteristics of a contractile actomyosin cable. Strikingly, we demonstrate that Ed is absent from certain cell types during development, generating endogenous interfaces between cells with and without Ed; these Ed expression borders display contractile features identical to those of *ed* mutant clones. In wild type ovaries, Ed is absent from one of the two cell types of the appendage primordium, generating an Ed expression border within the primordium. In the embryo, Ed becomes undetectable in the amnioserosa prior to dorsal closure, resulting in an Ed expression border that coincides with the well-characterized contractile actomyosin cable between these tissues. In both cases, elimination of Ed results in the absence of this contractile structure and in defective morphogenesis. Taken together, these data suggest that differential Ed expression between cell types induces the formation of a contractile actomyosin cable at their interface. These observations may identify a general morphogenetic mechanism that converts a difference in protein expression into a local effect on the cytoskeleton.

2.3 Materials and methods

2.3.1 *Drosophila* strains

The ed^{F72} allele was isolated in a genetic screen of mosaic females bearing

P{neoFRT}40A chromosomes that had been mutagenized with ethyl methanesulfonate. Other strains used were dec^{VA28}, *P{dec+}2L* (Nilson and Schupbach, 1998), ed^{lF20} *P{neoFRT}40A* (gift of J.-C. Hsu), *rho1.1* (Ip et al., 1992), *w; al dp P{NM}31E P{neoFRT}40A*, and *y w P{hsFLP}122; P{NM}31E P{neoFRT}40A*, sqh-GFP-moesin (SGMCA) (gift of D. Kiehart).

2.3.2 Mapping and identification of the F72 mutation

The F72 mutation was mapped through meiotic recombination between the parental strains *w; al dp P{NM}31E P{neoFRT}40A* and *w;* ed^{F72} *P{neoFRT}40A* using single nucleotide polymorphisms as molecular markers, as described previously (Berger et al., 2001; Hoskins et al., 2001; Martin et al., 2001). After mapping the F72 phenotype to an interval containing *ed*, all 9 *ed* exons, their splice sites, and approximately 1kb of flanking genomic DNA were amplified by PCR, in fragments of approximately 800 bp, and sequenced. The only difference between the F72 chromosome and the parental chromosome was an A to T substitution at position 1043 of the transcript, generating a premature stop at codon 205 of the *ed* open reading frame. The ed^{F72} allele is therefore predicted to encode a protein truncated in the second of seven immunoglobulin-like domains predicted by the ExPASy ScanProsite proteomics algorithm.

2.3.3 Generation of Ed antiserum

The cDNA RE66591 (*Drosophila* Genome Resources Center) was used as

template to amplify by PCR the fragment encoding the C-terminal domain of Ed, which was then cloned in frame into the pGEX2T-His_6 vector (gift of S. Gunderson). After expression in *E. coli* BL21 cells, the recombinant protein was purified by selection for the His_6 tag and used to immunize rats.

2.3.4 Mitotic recombination

Mitotic follicle cell clones were induced in females of the genotype *y w P{hsFLP}122; P{NM}31E P{neoFRT}40A/ed P{neoFRT}40A* by incubating pupae at 37ºC for 1 hour on three consecutive days. Prior to dissection, well-fed mosaic females were incubated at 37ºC for 80 minutes to induce expression of N-myc (NM) clone marker (Xu and Rubin, 1993). Germline clones homozygous for either ed^{F72} *or* ed^{lF20} were generated and imaged as described previously (Chou and Perrimon, 1996)(Schock and Perrimon, 2002a).

2.3.5 Immunohistochemistry

Fixation and staining of ovaries and embryos was performed as described previously (Van Buskirk and Schupbach, 2002; Wieschaus and Nusslein-Volhard, 1986). Antibodies used were anti-Ed (1:1,000 for ovaries, 1:10,000 for embryos), anti-c-Myc supernatant 9E10 (1:100, Developmental Studies Hybridoma Bank (DSHB)), anti-phospho-myosin light chain 1 Ser19 (1:250, Cell Signaling Technology), anti-DE-cadherin DCAD2 supernatant (1:100, DSHB), anti-Arm N2 7A1 supernatant (1:100 for

ovaries; 1:500 for embryos, DSHB), anti-β-galactosidase 40a1 supernatant (1:50, DSHB), anti-Broad Core supernatant (1:50, DSHB), anti-Enabled 5G2 (1:500, DSHB) and anti-phosphotyrosine (1:200, Upstate Cell Signaling Solutions). All secondary antibodies (Molecular Probes) were pre-blocked against ovaries and used at a final concentration of 1:500 for 2 hours at room temperature or 1:1,000 overnight at 4°C. For F-actin labeling, tissues were incubated for 2 hours at room temperature with 0.5 U/ml of Alexa Fluor 546 phalloidin (dried of methanol; Molecular Probes).

2.4 Results

2.4.1 Follicle cell clones homozygous for a novel mutation exhibit smooth borders

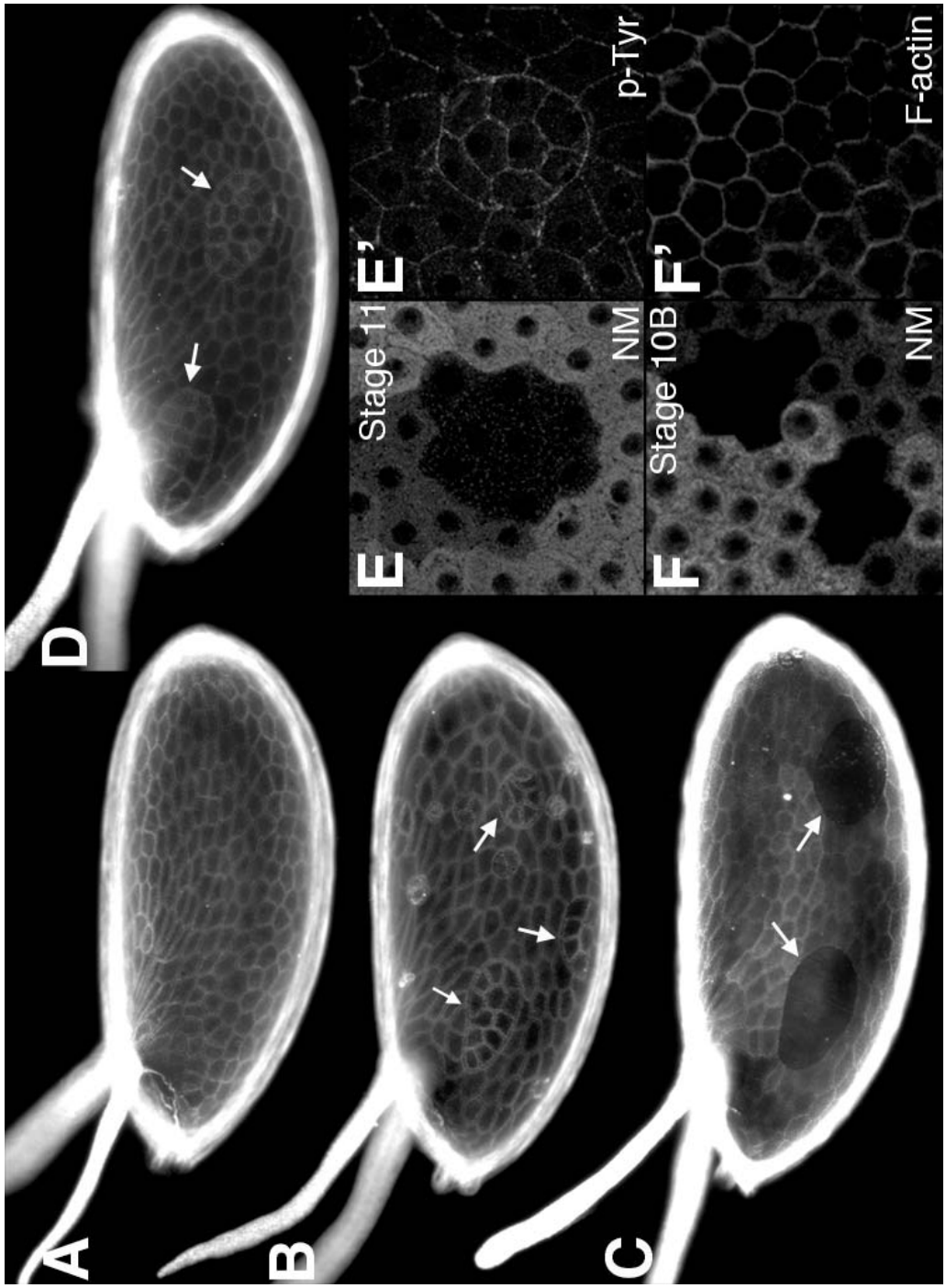
In a genetic screen for defects associated with follicle cell clones, we recovered a mutation, initially designated F72, with a novel effect on the organization of the imprints on the eggshell surface. The pattern of these imprints reflects the organization of the cells in the follicular epithelium, which secretes the eggshell and degenerates before the egg is laid (Figure 2.1A). Eggs produced by females bearing mitotic follicle cell clones homozygous for this mutation display subsets of eggshell imprints organized into groups with smooth borders (Figure 2.1B). When the F72 mutant clones were marked with the *defective chorion-1 (dec)* marker, which confers a distinct appearance on the eggshell secreted by the mutant cells (Hawley and Waring, 1988; Nilson and Schupbach, 1998; Wieschaus et al., 1981), the *dec*-marked imprints were contained exclusively within the smooth borders, indicating that these borders occur at the

interface of imprints produced by mutant and non-mutant cells (Figure 2.1C).

Consistent with the mosaic eggshell phenotype, clones of homozygous mutant follicle cells exhibit smooth borders with adjacent heterozygous or homozygous wild type cells. Interfaces between mutant cells within the clone, however, appear normal (Figure 2.1E,E'). Interestingly, the smooth clone border is detectable only at the apical side of the epithelium, while the basal aspect of the clone displays no obvious phenotype (compare Figure 2.1E and 1E'). This mosaic phenotype also exhibits a surprising temporal profile. The smooth clone border is completely penetrant in early stage egg chambers (data not shown) but, during stage 10 of mid-oogenesis, the border of F72 mutant clones becomes indistinguishable from adjacent intercellular interfaces (Figure 2.1F,F'). The disappearance of the phenotype is transient, however, and by stage 11 the marked smoothness of the clone border is again readily detectable and completely penetrant (Figure 2.1E,E'), and persists for the remainder of oogenesis.

Figure 2.1. F72 homozygous follicle cell clones produce groups of eggshell imprints with a smooth border.

In all figures, anterior is to the left and dorsal is at the top, unless otherwise indicated. **A.** Wild-type egg. **B.** Egg from an F72 mosaic female. Groups of surface imprints exhibit smooth borders (arrows). **C.** Egg from a female with homozygous F72 clones marked with the *dec* eggshell marker (arrows). **D.** Egg from an ed^{lF20} mosaic female. E,E'. Clone of F72 homozygous follicle cells in a stage 11 egg chamber. **E.** Clone is marked by the absence of the NM clone marker (basal confocal section); heterozygous and homozygous wild type cells are also visible. **E'.** Anti-phosphotyrosine (p-Tyr) staining, apical confocal section. The apical clone border is smooth. **F,F'.** Rhodamine-phalloidin staining to visualize filamentous actin (F-actin). F72 mutant clones at stage 10B (F, basal confocal section) do not exhibit a smooth border (F', apical confocal section).


A
B
C
D
E
Stage 11
NM
E'
p-Tyr
F
Stage 10B
NM
F'
F-actin

2.4.2 The F72 mutation generates a nonsense mutation in the *ed* gene

Meiotic mapping using single nucleotide polymorphisms as molecular markers located the F72 mosaic phenotype to a small interval containing exons 3, 4 and 5 of a single gene, *ed* (data not shown). Sequencing revealed a single nucleotide substitution, which generates a premature termination at codon 205 of the *ed* open reading frame. The identification of this nonsense mutation, together with the mapping of the phenotype to the same small interval, suggests that F72 is allelic to *ed*. Moreover, the F72 mutation fails to complement ed^{lF20}, an independently isolated *ed* allele that contains a premature termination at codon 63 (de Belle et al., 1993; Escudero et al., 2003). Follicle cell clones homozygous for ed^{lF20} exhibit a smooth border phenotype indistinguishable in all respects from F72 mutant clones (Figure 2.1D and data not shown), confirming that the phenotype is the result of the mutation in *ed*.

ed encodes a 1332 amino acid transmembrane protein with seven immunoglobulin (Ig) domains, a Fibronectin type III domain and a cytoplasmic tail with a PDZ-binding domain (see Materials and methods) (Bai et al., 2001; Wei et al., 2005). The ed^{F72} allele is predicted to encode an Ed protein that is truncated in the second Ig domain, thus lacking most of the extracellular domain as well as the transmembrane and intracellular domains and therefore unlikely to retain Ed function. The Ed extracellular domain resembles that of Ig-type cell adhesion molecules, and recently Ed has been reported to be a component of adherens junctions (Wei et al., 2005). The molecular

nature of Ed is therefore consistent with the *ed* mosaic phenotype, and suggests that the absence of this molecule from the cell surface affects the interaction between wild type and mutant follicle cells.

2.4.3 *ed* clone borders induce assembly of a contractile actomyosin cable

Our initial observations of *ed* mutant follicle cell clones revealed that the apical clone circumference is markedly reduced relative to the basal circumference. In addition, filamentous actin (F-actin) appears enriched at the apical interface between wild type and *ed* mutant cells (Figure 2.2A, A'), but not at the basal interface (Figure S2.1). Together with the reduced apical circumference (Figure 2.2B,B', and compare Figure1E to Figure 2.1E' and Figure 2.2A to Figure 2.2A'), this observation suggested the presence of a contractile actin cable at the clone border. Consistent with this hypothesis, the active, phosphorylated form of the light chain of non-muscle myosin II (p-MLC) is also enriched at the apical clone border (Figure 2.2C, C'), suggesting that non-muscle myosin II is activated at the interface between wild type and *ed* mutant cells (Sellers, 1991; Trybus, 1991). Closer inspection of the clone border resolves two "rings" of p-MLC immunoreactivity (Figure 2.2D, D'), raising the possibility that one contractile structure assembles within the *ed* mutant cells at the clone border and another in the adjacent wild type cells. Identical mosaic phenotypes were observed with both ed^{F72} and ed^{lF20}.

Figure 2.2. *ed* clone borders induce the formation of a contractile actin cable and reduction in adherens junction components.

A. ed^{F72} follicle cell clone (lack of NM marker, basal confocal section). **A'.** Rhodamine-phalloidin staining (apical confocal section) reveals enriched F-actin the clone border and reduced apical circumference. **B, B'.** Cross-section of an ed^{F72} mutant clone (B) stained with rhodamine-phalloidin (B') illustrates the apical constriction of the clone border (arrows, apical is toward the bottom). **C.** ed^{F72} follicle cell clone (basal confocal section). **C'.** Increased p-MLC immunoreactivity (apical confocal section) is detected at the clone border. **D, D'.** Enlargement of an ed^{F72} clone border. Two parallel lines of p-MLC immunoreactivity can be resolved. **E, E.'** (Stage 12). Some ed^{F72} mutant clones exhibit severely reduced or discontinuous DE-cad immunoreactivity at the clone border (arrow). **F, F'.** (Stage 12). At ed^{F72} mutant clone borders with a milder DE-Cad defect, DE-Cad is occasionally absent (arrow) but often discontinuous or unaffected (open and closed arrowheads, respectively).

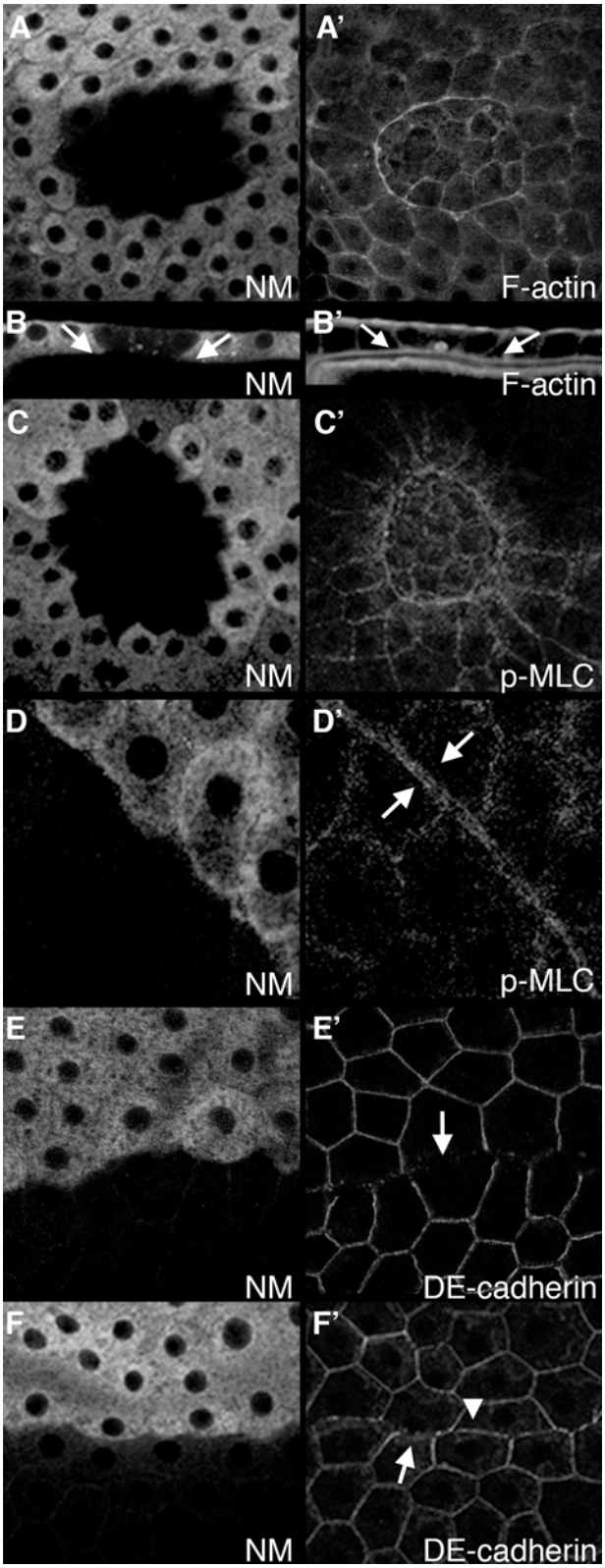
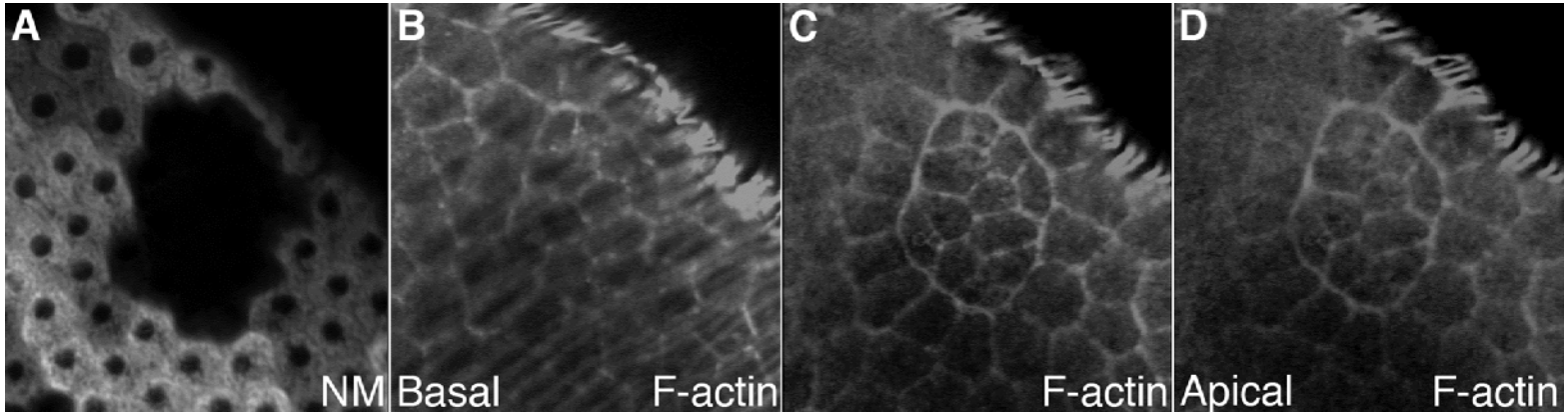

A
NM
A'
F-actin
B
NM
B'
F-actin
C
NM
C'
p-MLC
D
NM
D'
p-MLC
E
NM
E'
DE-cadherin
F
NM
F'
DE-cadherin

Figure S2.1. F-actin is enriched apically but not basally at the border of *ed* mutant clones.

A. Clone of follicle cells homozygous for ed^{F72} (lack of NM marker, basal confocal section). **B-D.** Rhodamine-labeled phalloidin staining (basal to more apical confocal sections) reveals enriched F-actin at the apical clone border.


A
NM
B
Basal
F-actin
C
F-actin
D
Apical
F-actin

The reduced circumference and enrichment of F-actin and p-MLC at the apical *ed* clone border are characteristic of the supracellular contractile actomyosin structures that mediate the epithelial movements observed in processes such as wound healing and embryonic epithelial closure (Bement, 2002; Martin and Parkhurst, 2004). We propose that the juxtaposition of wild type and *ed* mutant cells is sufficient to trigger the assembly of such a structure at their interface, resulting in a contractile force at the border that generates the apically-constricted smooth circumference of *ed* mutant follicle cell clones. This phenotype is similar to that reported for *ed* mutant clones in the wing imaginal disc epithelium (Wei et al., 2005). However, while *ed* mutant cells in the wing disc required a genetic growth advantage to recover sufficient clones for analysis, *ed* mutant follicle cells display no detectable defects in growth or viability. This difference may reflect tissue specificity in the requirement for *ed*, or a difference in the *ed* mutant chromosome studied in the wing.

2.4.4 Adherens junctions are destabilized at the border of *ed* mutant clones

Because the smooth border of *ed* mutant follicle cell clones suggested possible differential adhesion with the neighboring wild type cells (Lawrence, 1997)(Dahmann and Basler, 1999; Tepass et al., 2002), we determined whether *ed* mutant clones exhibit altered levels of the cell adhesion molecule DE-cadherin (DE-cad). At stage 10, when the smooth border phenotype is not detectable, the level and distribution of DE-cad appeared normal at the border of 38/38 *ed* mutant clones examined (data not shown). After stage 11, DE-cad immunoreactivity was strongly reduced or absent at the clone

border in 6/20 clones observed (Figure 2.2E,E'). This effect was less dramatic in 11/20 clones (Figure 2.2F,F'), where DE-cad at individual interfaces along a single clone border appeared either undetectable (Figure 2.2F', arrow), wild type (Figure 2.2F', arrowhead), or discontinuous (Figure 2.2F', open arrowhead). In 3/20 clones, there was no detectable effect. Levels of Armadillo (Arm), the *Drosophila* homolog of ß-catenin and an intracellular component of adherens junctions, were similarly affected (data not shown). In both cases, the degree of disruption did not correlate with clone size or position, and was variable even between egg chambers of the same stage. These data indicate that the juxtaposition of wild type and *ed* mutant cells can affect the distribution of DE-cad and Arm. However, given the variability of this effect, it remains unclear whether alteration of adherens junction components is the cause of the *ed* smooth border phenotype.

2.4.5 Ed exhibits a dynamic expression pattern in the follicular epithelium

To visualize the distribution of Ed in the follicular epithelium, we generated an antiserum against the Ed intracellular domain. We detected no immunoreactivity in *ed* mutant follicle cells, confirming the specificity of the antiserum (Figure 2.3A,A'). In individual follicle cells, Ed levels appear highest apically with lower levels detectable in lateral membranes (Figure 2.3B, inset), resembling the distribution of DE-cad and Arm and consistent with recent evidence implicating Ed as an adherens junction component (Wei et al., 2005). As reported previously, Ed immunoreactivity is either discontinuous (Figure 2.3A, arrowhead) or absent (Figure 2.3A, arrow) from the surrounding wild type

cells at the interface with *ed* mutant cells, consistent with previous observations that Ed molecules on adjacent cells can interact homophilically (Islam et al., 2003; Spencer and Cagan, 2003).

Ed exhibits a spatially and temporally dynamic expression pattern in wild type ovaries. In early stages of oogenesis, similar levels of Ed are detectable in all follicle cells (Figure 2.3B, left), but begin to decline by stage 8 (Figure 2.3B, middle). By early stage 10B, little or no Ed is detectable above background levels (Figure 2.3B, right). Interestingly, the absence of detectable Ed at this stage coincides with the transient disappearance of the *ed* mosaic phenotype, supporting the hypothesis that the smooth border of *ed* mutant clones is triggered by the juxtaposition of cells with and without Ed; presumably *ed* mutant clones do not exhibit a smooth border at this stage (see Figure 2.1F,F') because Ed is also absent from the surrounding wild type cells.

Figure 2.3. Ed exhibits a spatially and temporally dynamic distribution during oogenesis.

A. Homozygous ed^{F72} follicle cell clone (lack of NM marker, basal confocal section). **A'.** Ed immunoreactivity is absent from the *ed* mutant cells and absent (arrow) or discontinuous (arrowhead) from wild type cells at the clone border (apical confocal section). **B.** Ed is enriched at the apical (germline-facing) side of the tissue (inset; apical is indicated with an arrowhead, basal with an arrow) at early stages (left) then becomes undetectable by early stage 10B (right). **C.** Late stage 10B/early stage 11 (dorsal view). Ed is detected at the dorsal midline of the main body follicle cells (only the posterior half of the egg chamber is shown). **C'.** Same as C, labeled with rhodamine-conjugated phalloidin. A smooth interface coincides with the endogenous Ed expression border (arrow). **D.** Stage 11 (dorsolateral view). Ed is absent from two dorsolateral groups of follicle cells. **D'.** Same as D (dorsal anterior portion), labeled with rhodamine-conjugated phalloidin. A smooth interface (arrow, arrowhead) corresponds to the endogenous Ed expression border.

A
NM
A'
B
Ed
C
Ed
C'
F-actin
D
Ed
D'
F-actin

Also consistent with the temporal profile of the *ed* mosaic phenotype, Ed immunoreactivity reappears in late stage 10B and persists throughout the remainder of oogenesis. In late stage 10B, Ed is present in a T-shaped pattern along the dorsal anterior midline of the epithelium (Figure 2.3C). Later, beginning in stage 11, Ed is present in all main body follicle cells except for two dorsolateral populations that exhibit no detectable Ed (Figure 2.3D). The border of an *ed* mutant clone is not smooth when it falls within one of these two populations, providing functional evidence for the absence of Ed (data not shown). During stage 12, Ed becomes detectable in this domain (data not shown).

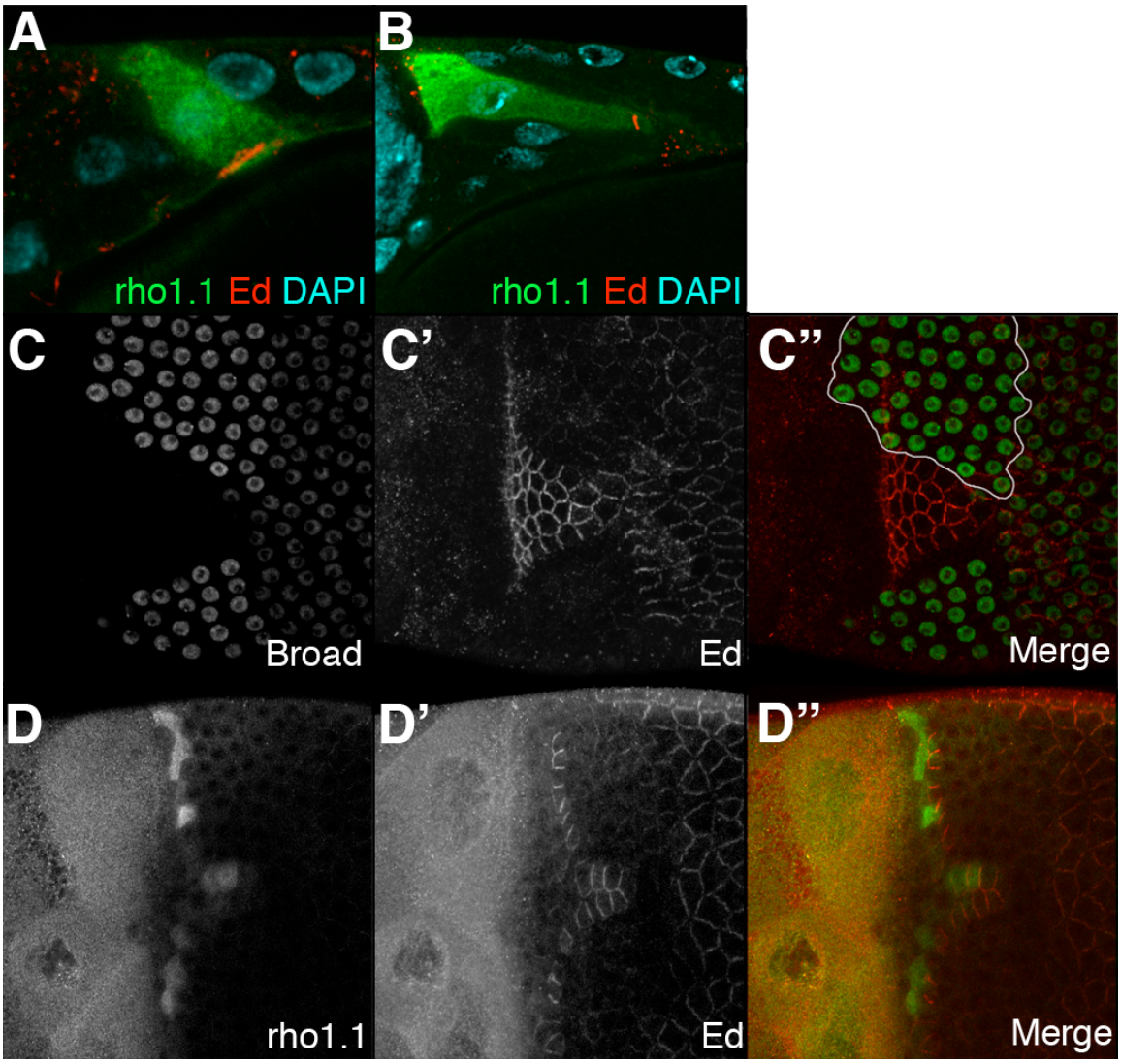
The absence of Ed from specific follicle cell populations generates endogenous interfaces between cells with and without Ed, which we refer to as Ed expression borders, that resemble the ectopic interfaces generated by *ed* mutant clones. Indeed, we found that these endogenous Ed expression borders are smooth and exhibit the same apical enrichment of F-actin and p-MLC associated with *ed* mutant clones (Figure 2.3C',D', and data not shown). At later stages, however, the enrichment of actin at this endogenous border appears less pronounced due to increased F-actin levels in the individual cells within this domain (Figure 2.3D'). These data further support the hypothesis that juxtaposition of cells with and without Ed results in formation of a contractile actin cable at their interface. Moreover, the spatial and temporal regulation of the appearance of this Ed expression border suggested that it may have a developmental function.

2.4.6 Ed is absent from the roof cells of the appendage primordia

The dorsal-anterior Ed expression pattern suggested a correlation between Ed expression and the specification or behavior of the dorsal appendage primordia, which are located in the same region of the epithelium. Each primordium consists of two cell types, which are specified coordinately during midoogenesis. The majority of the cells in the primordium will form the roof of the appendage-producing tube, while the cells in a single "L"-shaped row at the anterior and medial edges of the primordium will form the tube floor. The roof cells are distinguished by high nuclear levels of the Broad protein, a zinc-finger transcription factor (Bayer et al., 1996; Deng and Bownes, 1997; DiBello et al., 1991; Tzolovsky et al., 1999), while the floor cells express a *lacZ* reporter driven by a portion of the *rhomboid* promoter (*rho1.1*) (Dorman et al., 2004; Ip et al., 1992; Ward and Berg, 2005). The *rho1.1* marker is detectable throughout the floor cell cytoplasm and thus also highlights the changes in floor cell shape that occur during appendage morphogenesis (Figure 2.4A,B).

Figure 2.4. Absence of Ed from the roof cells generates an endogenous Ed expression border.

A, B. Cross-sections (lateral view) through the dorsal anterior follicular epithelium at early (A) and late (B) stage 11. Ed (red) is detected in cells expressing the *rho1.1* floor cell marker (green). The floor cell shown in A has begun to elongate posteriorly; this elongation is more pronounced by the stage shown in B. Nuclei are shown in blue. **C-C".** Stage 11 (dorsal view). C. High Broad levels mark the roof cell nuclei. C'. Ed is present in all follicle cells except for two dorsolateral domains. C". Merge. Ed (red) is absent from the roof cells (green); one roof cell domain is outlined. **D-D".** Stage 11 (dorsal view). The two L-shaped floor cell domains (D, basal confocal section) align with the limit of the Ed domain (D', apical confocal section). D". Merge of Ed (red) and *rho1.1* (green). The patterns in merged images (C",D") are slightly out of register due to the different planes of the images and the onset of morphogenesis (see panel A).


A
rho1.1 Ed DAPI
B
rho1.1 Ed DAPI
C
Broad
C'
Ed
C''
Merge
D
rho1.1
D'
Ed
D''
Merge

We found that the follicle cell domains that lack Ed expression (see Figure 2.3D) coincide precisely with the two roof cell populations, which express high levels of Broad (Figure 2.4C-C"). In addition, visualization of the *rho1.1* floor cell marker revealed that Ed is present apically in the adjacent floor cells (Figure 2.4A,B,D-D"). The border of the domain lacking Ed therefore aligns precisely with the roof/floor interface, which has been observed previously to be smooth (Ward and Berg, 2005), Our data show that an endogenous Ed expression border corresponds to a contractile interface between the two cell types that populate the appendage primordium.

The dorsal-ventral pattern of follicle cell fates, including both the appendage primordia and the dorsal midline, is established through dorsally localized activation of the *Drosophila* epidermal growth factor receptor (Egfr) pathway (Berg, 2005; Nilson and Schupbach, 1999; Roth, 2003), and Ed has been reported to downregulate Egfr signaling in the eye imaginal disc (Bai et al., 2001; Rawlins et al., 2003b; Spencer and Cagan, 2003). However, large *ed* mutant follicle cell clones encompassing the dorsal midline and one or both appendage primordia (n=6) have no detectable effect on the pattern of Broad expression (data not shown). This observation indicates that Ed functions downstream of cell fate determination and does not regulate Egfr signaling in this patterning process.

2.4.7 *ed* is required for morphogenesis of the appendage primordia

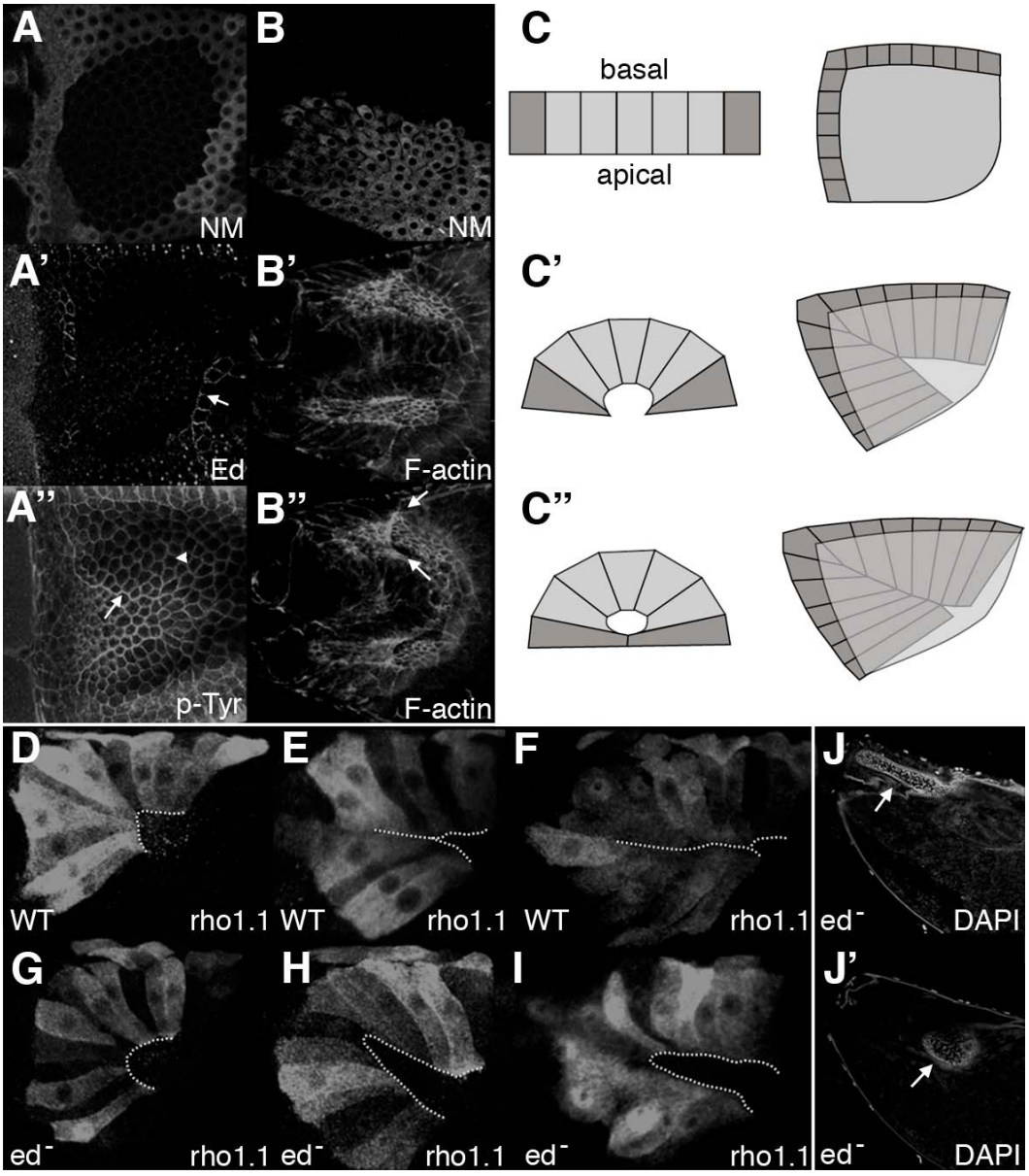
The contractile features of endogenous and ectopic Ed expression borders, suggested a potential function for the endogenous Ed expression border in appendage tube morphogenesis. We first asked whether differential Ed expression is required for the smooth border of the roof cell domain. In wild type stage 11 epithelia, a smooth border is detectable along the entire circumference of the roof cell domain, both at the roof cell/floor cell interface (see Figure 2.3D', arrow) and at the posterior of the roof cell domain (see Figure 2.3D', arrowhead). When the endogenous Ed expression border was eliminated at this stage by a large *ed* mutant follicle cell clone (Figure 2.5A,A'), the smooth border at the posterior of the roof cell domain was abolished in 7/7 primordia recovered (Figure 2.5A", arrowhead). Some delineation between the roof and floor cell populations was still visible, but this interface was not smooth (Figure 2.5A", arrow).

To determine whether the endogenous Ed expression border is required for the remodeling of the appendage primordia into epithelial tubes we analyzed *ed* mosaic egg chambers at later stages, when the tubes are being formed. In mosaic epithelia with one mutant (Figure 2.5B, top) and one wild type primordium (Figure 2.5B, bottom), the nascent *ed* mutant tube was shorter (Figure 2.5B') and exhibited a wider opening (Figure 2.5B'', arrows) than the wild type tube, suggesting a defect in tube morphogenesis in the absence of an Ed expression border (n=3).

Figure 2.5. Ed is required for tube floor closure during appendage morphogenesis.

A. Stage 11 mosaic egg chamber (dorsal view) with a large *ed* mutant clone (lack of NM marker) that includes much of both appendage primordia. **A'.** Ed expression is detectable in the posterior non-mutant follicle cells (arrow), indicating that this egg chamber is of a stage at which Ed would be expressed in all follicle cells except the presumptive roof cells (see Figure 2.3D). **A".** Anti-phosphotyrosine staining. The interface between the roof and floor cell domains is morphologically distinguishable but not smooth (arrow), and the posterior border of the roof cell domain (arrowhead) is not distinguishable. **B.** Stage 12 egg chamber (dorsal view) with one wild type (bottom) and one mutant (top, lack of NM marker) appendage primordium. **B', B".** Rhodamine-phalloidin staining, basal section (B'), apical confocal section (B"). The opening of the mutant tube appears wider (arrows). **C-C".** Diagram of a single appendage primordium at successive stages of tube formation. Both cross-section (left) and surface views (right; anterior to the left, dorsal to the top) are shown. The presumptive roof cells (light grey) are flanked anteriorly and medially by a single row of floor cells (dark grey). To emphasize the floor cell movements, roof cells are not delineated individually in the surface views. **D-I.** Appendage primordia expressing the *rho1.1* floor cell marker. D-F. Wild type appendage primordia. D. Primordium at onset of tube extension phase (floor cell domain aligned with oocyte/nurse cell margin). The floor cells have elongated and the apices nearest the "hinge" between the anterior and medial domains have met. E. Tube extension phase (floor cell domain overlaps nurse cells). The tube floor is nearly fully closed. F. Later stage than E. The tube floor is closed. G-I. *ed* mutant primordia. G. Same stage as D. The floor cell apices have not met. H. Tube extension stage,

comparable to E. The floor cell apices have not met; the tube floor remains open. I. Later stage than H. The tube floor remains open. **J, J'.** Two sides of a single stage 14 egg chamber in which all follicle cells are mutant for *ed*. Autofluorescence and DAPI staining reveal the eggshell structure and nuclei of the follicular epithelium, respectively. The dorsal appendages are severely reduced (arrow).


A
B
C
basal
apical
NM
NM
A'
B'
C'
Ed
F-actin
A''
B''
C''
p-Tyr
F-actin
D
E
F
J
WT
rho1.1
WT
rho1.1
WT
rho1.1
ed-
DAPI
G
H
I
J'
ed-
rho1.1
ed-
rho1.1
ed-
rho1.1
ed-
DAPI

We further characterized this tube defect using the *rho1.1* reporter to visualize the floor cell movements that occur during tube formation. In wild type primordia, at the onset of tube morphogenesis the floor cells elongate and the apices of the anterior and medial floor cell domains begin to approach each other, moving underneath the roof cells, which constrict apically at this stage (Figure 2.5C,C'; see Figure 2.4A,B). The floor cell apices meet first at the intersection (or hinge) between the anterior and medial domains and progressively converge, closing the tube floor (Figure 2.5C',C'',D-F). As floor closure nears completion, the wedge-shaped floor cell domain (Figure 2.5D) adopts a more rounded appearance (Figure 2.5E,F) (Dorman et al., 2004), and moves anteriorly. By the time the floor cell domain begins to overlap the nurse cell cluster, the tube floor is closed. The completed tube then continues to extend anteriorly.

In all phases of tube morphogenesis, the floor cells of both wild type (Figure 2.5D-F) and *ed* mutant (Figure 2.5G-I) primordia elongate to a similar degree and project their apices toward the future tube floor midline, indicating that this change in floor cell shape is Ed-independent. However, tube floor closure is defective in the absence of Ed. Prior to the tube extension phase, floor closure was partially complete in 8/38 wild type primordia but in 0/19 *ed* mutant primordia (Figure 2.5D,G). During tube extension, the tube floor was closed in 37/37 wild type primordia observed (Figure 2.5E,F). However, in *ed* mutant primordia the tube floor remained open in 16/17 cases observed (Figure 2.5H,I), indicating that the presence of Ed is required for proper tube floor closure.

This tube formation defect results in abnormal eggshell appendages. Appendage morphology can be visualized in egg chambers at stage 14; this is the final stage of oogenesis, when egg chambers have completed dorsal appendage formation. We examined stage 14 egg chambers from *ed* mosaic females, focusing on those that retained an intact follicular epithelium and lacked detectable Ed. Of 14 *ed* mutant appendages recovered, 12 were severely reduced in length or had failed to extend from the main body of the eggshell (Figure 2.5J,J'). These mutant appendage phenotypes confirm that the defects in floor closure observed in the absence of *ed* result in improperly formed epithelial tubes.

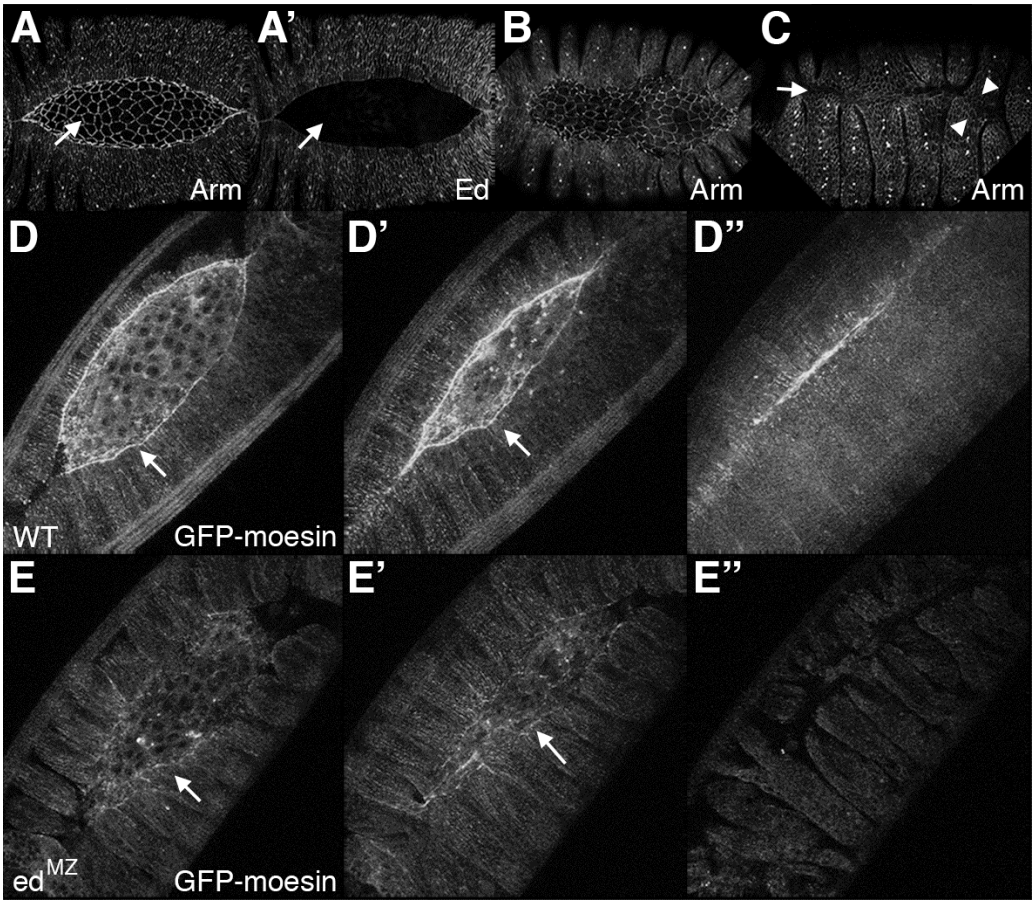
2.4.8 Differential Ed expression promotes embryonic dorsal closure

The morphogenetic movements of the floor cells are reminiscent of those observed during embryonic dorsal closure, where the lateral epidermal sheets of the embryo move dorsally and ultimately fuse at the embryonic dorsal midline, covering the extraembryonic amnioserosa (Jacinto et al., 2002; Martin and Parkhurst, 2004). Interestingly, the leading edge cells of the lateral epidermis assemble a supracellular contractile actomyosin cable that provides one of the forces driving epithelial closure (Young et al., 1993)(Hutson et al., 2003; Kiehart et al., 2000). Given the contractile nature of endogenous and ectopic Ed expression borders in the follicular epithelium, we asked whether the actomyosin cable between the amnioserosa and lateral epidermis is also associated with differential Ed expression. We found that during dorsal closure Ed is present in the lateral epidermis but undetectable in the amnioserosa (Figure 2.6A,A'), generating an endogenous Ed expression border at the interface of these two cell types.

To determine whether this differential Ed expression is necessary for the generation of the actomyosin cable and for dorsal closure, we generated embryos lacking both maternal and zygotic contributions of *ed* (ed^{MZ}). Fixed ed^{MZ} embryos exhibit apparent irregularities in the progression of the leading edge during dorsal closure stages (14/14; Figure 2.6B), as well as gaps and segment misalignments at the dorsal midline in later stage embryos (14/14; Figure 2.6C).

Figure 2.6. Ed is required for dorsal closure.

A, A'. Stage 14 embryo, dorsal view, zippering phase of dorsal closure (Jacinto et al., 2002). A. Arm immunoreactivity highlights the amnioserosa (arrow) and surrounding lateral epidermis. A'. Ed immunoreactivity is absent from the amnioserosa (arrow). **B,C.** Arm staining of ed^{MZ} mutant embryos at the zippering phase (B) and termination phases (C) of dorsal closure. Note the gaps along the dorsal midline (arrow) and misaligned segments (arrowheads). **D-D".** Successive images of a live wild type embryo expressing GFP-moesin during zippering (D, D') and termination (D"). Accumulation of GFP-moesin at the leading edge highlights the contractile cable (D, D', arrows). **E-E".** Live imaging of dorsal closure in an ed^{MZ} embryo expressing GFP-moesin. Note the absence of GFP-moesin accumulation at the leading edge (E, E', arrows), and the gaps and segment misalignment in the termination phase (E").


A
A'
B
C
Arm
Ed
Arm
Arm
D
D'
D''
WT
GFP-moesin
E
E'
E''
ed^MZ
GFP-moesin

We also used time-lapse confocal microscopy to image live wild type and ed^{MZ} embryos expressing a transgene encoding the actin binding fragment of *Drosophila* moesin fused to GFP (GFP-moesin), a well-characterized marker that labels F-actin (Edwards et al., 1997). In wild type embryos during dorsal closure, this marker highlights the actin cable at the interface of the lateral epidermis and amnioserosa (Figure 2.6D,D', arrows) and allows visualization of the progressive dorsal movement of the epidermis in live embryos (Figure 2.6D-D'') . In contrast to wild type embryos, ed^{MZ} embryos during dorsal closure stages fail to exhibit pronounced accumulation of GFP-moesin at the leading edge of the lateral epidermis (Figure 2.6E,E', arrows), suggesting that the actomyosin cable fails to assemble. Moreover, in these mutant embryos, the dorsal epidermis appears to buckle toward the amnioserosa, suggesting that a lack of tension prevents the formation of a taut interface with the amnioserosa (Grevengoed et al., 2001). As development proceeds, these embryos exhibit defective dorsal closure. The dorsal movement of the lateral epidermis is delayed compared to wild type embryos, and discontinuities and puckering at the dorsal midline and misalignment of opposing segments are ultimately observed. These data support the hypothesis that the Ed expression border is required for assembly of the supracellular actomyosin cable, and that the absence of this structure leads to defective morphogenesis.

2.5 Discussion

2.5.1 Ed expression borders assemble a contractile actomyosin structure that mediates epithelial morphogenesis.

We demonstrate that the borders of *ed* mutant follicle cell clones display a reduced apical circumference and apical enrichment of F-actin and p-MLC, suggesting that the juxtaposition of follicle cells with and without Ed is sufficient to trigger the assembly of an apical actomyosin cable at their interface. Based on these observations, we propose that the smooth, constricted border of *ed* mutant clones is the result of a contractile force generated by this structure. Consistent with this interpretation, *ed* clone borders do not exhibit this phenotype if the adjacent wild type cells, due to their position or developmental stage, also lack Ed. Thus the generation of this contractile structure is a result of an interface between cells with and without Ed, rather than the loss of Ed *per se*.

The apical constriction associated with the loss of Ed appears to be restricted to the Ed expression boundary itself; individual *ed* mutant follicle cells that do not contact the clone border do not display pronounced apical constriction. While the apical circumference of follicle cells in the interior of *ed* mutant clones occasionally appears reduced (see Figure 2.2A',C'), this effect is not observed in larger clones (see Figure 2.2E',F'). The reduction of apical circumference observed in individual *ed* mutant cells may therefore be a secondary consequence of the contractile force generated at the clone border, rather than a direct effect of the absence of Ed.

While a smooth border has been reported previously for *ed* mutant clones in the wing imaginal disc (Wei et al., 2005), our data are the first to reveal a developmentally regulated absence of Ed in specific cell types associated with epithelial sheet movements. We show that Ed is absent from the presumptive roof cells of the appendage primordia prior to tube morphogenesis, and from the embryonic amnioserosa prior to dorsal closure. In both cases, the resulting endogenous Ed expression borders are smooth and display features of a contractile actomyosin cable, and loss of Ed results in defects in epithelial closure. Because generation of ectopic Ed expression borders is sufficient to generate a smooth contractile intercellular interface, we interpret these defects as a result of the elimination of the endogenous Ed expression borders between these tissues. We propose that the juxtaposition of cells with and without Ed at these endogenous interfaces induces local contractility of the actin cytoskeleton that in turn drives the convergence of opposing epithelial domains during morphogenesis.

Ed does not appear to play a role, however, in the generation of the actin-rich smooth interface observed at the boundary between dorsal and ventral compartments of the wing imaginal disc (Major and Irvine, 2005). Differential expression of Ed between dorsal and ventral compartments is not detected, and *ed* mutant clones in either compartment exhibit smooth borders (Rawlins et al., 2003a; Wei et al., 2005). Therefore, despite a general morphological similarity, differential Ed expression does not appear to play a role at this epithelial boundary.

2.5.2 Multiple forces contribute to morphogenesis

While our data demonstrate that differential Ed expression generates a contractile interface that is required for proper appendage tube formation and dorsal closure, other forces also contribute to these processes. The involvement of multiple forces is best understood for dorsal closure where, in addition to the contractile actin cable at the epidermis/amnioserosa interface, apical constriction of the individual amnioserosa cells also drives the movement of the leading edge, particularly in the initial stages of the process. In later stages, interactions between filopodia of opposing leading edge cells also contribute to the completion of closure (Jacinto et al., 2002; Kiehart et al., 2000). Consistent with the involvement of multiple forces, the lateral epidermal edges do ultimately approach the dorsal midline in $ed^{M/Z}$ embryos, suggesting that the elimination of the Ed expression border specifically disrupts the actin cable, while the other forces remain functional.

The cell movements and shape changes associated with the morphogenesis of the appendage primordia appear very similar to those observed in dorsal closure. In addition to the convergence of opposing floor cell domains to form the tube floor, the individual roof cells constrict apically (Dorman et al., 2004), similar to the amnioserosa cells. This roof cell behaviour is likely a consequence of roof cell fate determination rather than the absence of Ed, since *ed* mutant cells outside of this domain do not exhibit this same pronounced reduction in apical circumference. Presumably the epithelial groove generated by the coordinated apical constriction of the roof cells, together with the elongation of the floor cells, can generate the rudimentary tubes that give rise to the

severely shortened and malformed appendages observed in the absence of floor closure in *ed* mutant primordia.

Given the proposed role of Ed as a homophilic adhesion molecule (Islam et al., 2003; Rawlins et al., 2003a; Spencer and Cagan, 2003), selective affinity may also contribute to morphogenesis. For example, as the anterior and medial floor cells elongate toward the midline of the primordium, preferential affinity for the opposing floor cells, which also express Ed, over the roof cells, which lack Ed, may favor floor cell association. In dorsal closure, Ed-mediated interactions between opposing leading edge cells could play a similar role. It is also possible that differential Ed expression may have a dual function, contributing to morphogenesis through generation of both a contractile interface and differential affinity between cell types.

2.5.3 *ed* mutant follicle cells do not undergo premature cell death

In irradiated cultured epithelia, a smooth contractile interface has been observed between apoptotic epithelial cells and their neighbors, suggesting that active extrusion of dying cells preserves the integrity of the epithelium (Rosenblatt et al., 2001). This effect resembles the *ed* mosaic phenotype, but the presence on the eggshell surface of imprints produced by *ed* mutant cells indicates that these cells do not die before the secretion of eggshell at the end of oogenesis. Moreover, *ed* mutant clones are not detectably smaller than their associated twin spots and we detect no evidence of DNA fragmentation or the active form of the proapoptotic enzyme caspase-3 in *ed* mutant

follicle cells (data not shown), confirming that the contractile border of *ed* mutant clones is not induced by premature cell death.

2.5.4 Ed expression borders affect adherens junction components

We have observed reduced levels and altered distribution of DE-cad and Arm at the border between cells with and without Ed. In contrast, the distribution and level of DE-cad and Arm at the interfaces between *ed* mutant follicle cells within a clone appear normal. This observation demonstrates that, although recent evidence suggests that Ed is a component of adherens junctions (Wei et al., 2005), Ed is not generally required for adherens junction stability.

A border effect on adherens junction components has also been reported in *ed* mutant clones in the wing disc epithelium, where it has been proposed to play a causative role in the generation of a smooth clone border by mediating cell sorting (Wei et al., 2005). However, at the border of *ed* mutant follicle cell clones this effect is frequently mild and occasionally undetectable, while the contractile phenotype is completely penetrant. This difference could suggest that a functionally relevant alteration in adherens junction distribution is only occasionally reflected by diminished immunoreactivity. Alternatively, this effect on adherens junction components could be instead a consequence of contraction of the actin cable assembled at the Ed expression border. Indeed, an actomyosin-based contractile force has been proposed to be capable of disrupting adherens junctions (Bertet et al., 2004; Sahai and Marshall, 2002). However, we have not observed a disruption of adherens junction components at

endogenous Ed expression borders (data not shown), raising the possibility that this effect is not involved in Ed expression border function.

2.5.5 Local effect of Ed expression borders on the actin cytoskeleton

How an Ed expression border induces the local assembly of a contractile actin cable remains unclear. A potential connection between Ed and the actin cytoskeleton is suggested by the reported interaction between Ed and Canoe (Cno), which is homologous to mammalian Afadin and contains a actin filament binding domain, suggesting that Ed may function as a Nectin, the Afadin binding partner (Takai and Nakanishi, 2003; Wei et al., 2005). However, in *ed* mosaic wing imaginal discs, Cno distribution is altered throughout *ed* mutant clones, not just at the border (Wei et al., 2005). This observation does not exclude a role for Cno in Ed function but, because this effect on Cno is not restricted to the clone border, it alone cannot explain the localized effect on the actin cytoskeleton. Interestingly, an interaction with Ed does not appear to be strictly required for proper membrane localization of Cno, since Ed is lost from the amnioserosa during dorsal closure while Cno remains detectable (Boettner et al., 2003)(Takahashi et al., 1998).

An obvious distinguishing feature of Ed expression borders is the absence of Ed from the apposing face of the Ed-expressing population, presumably due to the absence of trans homophilic interactions. The mechanism that removes or redistributes Ed from this interface, rather than the absence of Ed itself, might therefore mediate the border-specific effect on the actin cytoskeleton. If the machinery that removes Ed, for example

through endocytosis, is not completely specific, such a model could also account for altered levels of DE-cad and Arm at these interfaces. Alternatively, the absence of homophilic interactions across Ed expression borders could favor the interaction of Ed with other factors, which could in turn mediate border specific effects.

2.6 Conclusions

Our data demonstrate a novel role for Ed in epithelial morphogenesis. In both the follicular epithelium and the embryo, the juxtaposition of follicle cells with and without Ed is sufficient to trigger local assembly of a contractile actin cable, and the defective epithelial closure observed in the absence of Ed suggests that this structure drives cell movements. This work identifies an Ed expression border as a functional entity in this process, and demonstrates the existence of a mechanism that functions downstream of cell fate determination to convert a difference in protein expression between two cell types into a local effect on the cytoskeleton that drives epithelial movements and mediates morphogenesis. We propose that differential Ed expression between two cell populations may represent a general mechanism for regulating tissue movements.

2.7 Acknowledgements

We thank Celeste Berg, Jui-Chou Hsu, Dan Kiehart and Samuel Gunderson for reagents, Danny Brody and Olivia Alder for assistance with meiotic mapping, and Christina Barkauskas for help with the initial identification of the F72 mutation. We also thank Lucía Cáceres, Alan Fanning, Paul Lasko, Craig Mandato and Jocelyn Moore for helpful comments on the manuscript. Monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. This work was supported by the Natural Sciences and Engineering Research Council of Canada.

2.8 The differential expression of Ed triggers the formation of actomyosin cables during epithelial morphogenesis

The differential expression of Ed in mosaic follicular epithelia triggers the formation of actomyosin cables at the clones interface. In addition, groups of cells downregulate their expression of Ed prior to morphogenesis thus generating endogenous differential expression of Ed interfaces. Since an actomyosin cable assembles at such interfaces, this work prompted the hypothesis that the differential expression of Ed between two neighbouring tissues results in the formation of an actomyosin cable at their interface.

Yet, a few questions remain unresolved. Can the assembly of the actomyosin cable be prevented by the addition of Ed in the tissue that normally downregulates Ed expression, i.e. the amnioserosa? Is the loss of a homophilic binding partner at one face of the Ed expressing cell, where it contacts an Ed lacking cell, responsible for the formation of an actomyosin cable? Nonetheless, the assembly of the actomyosin cable at the interface of cells with and without Ed represents a particularly interesting quandary; how can the differential expression of a cell adhesion molecule trigger the formation of a multicellular contractile actin structure in a planar polarized fashion? We know that certain actin regulators including Dia and Ena accumulate at the leading edge of the epidermis where the actomyosin cable assembles (Gates et al., 2007; Homem and Peifer, 2008). Is the asymmetric distribution of Ed around the cells of the leading edge responsible for the polarized accumulation of such actin regulators at the leading edge?

2.8.1 The molecular function of Echinoid

Little is known about the molecular function of Ed. Recent work has focused mainly on the only known motif in the cytoplasmic tail of Ed, a PDZ domain binding motif located at the very carboxy-terminus, which acts as an anchor for PDZ domain containing proteins. The combined effort of different studies has proposed multiple PDZ containing partners for Ed: Baz, Canoe (Cno; the *Drosophila* homologue of Afadin), Jaguar/MyosinVI and the *Drosophila* Glutamate Receptor Interacting Protein (DGrip) (Lin et al., 2007; Swan et al., 2006; Wei et al., 2005). These different interaction partners suggest that Ed is either a very promiscuous protein in its interactions or perhaps that each different interaction is specific to a particular function of Ed at a certain time and in a certain tissue specific.

Most relevant to this work are the interactions between Ed and both the actin binding protein Cno and the polarity protein Baz. It was shown through *in vitro* binding assays and co-immunoprecipitation of whole tissues that Ed can interact with either Baz or Cno and that the PDZ domain binding motif of Ed is required for these interactions. As Baz can interact with either Ed or Armadillo, it was concluded that Ed cooperates with DE-Cadherin in cell adhesion and that they redundantly position Baz to adherens junctions. The intimate ties between adherens junction stability and the regulation of the actin cytoskeleton thus prompted the hypothesis that the role of Ed as a modulator of adherens junction stability can influence the formation of actomyosin cables.

This model raises multiple issues that need to be settled. First, although the differential expression of Ed can cause the adherens junctions to appear discontinuous at *ed* mutant clone borders, this phenotype is mildly penetrant in mosaic follicular epithelia and is not detectable at endogenous Ed interfaces in the appendage tube primordium or at the interface between the epidermis and amnioserosa during dorsal closure (Kaltschmidt et al., 2002; Laplante and Nilson, 2006). Moreover, the loss of Cno in *ed* mutant wing imaginal disc clones has not been observed in *ed* mutant embryos using different alleles of *ed* suggesting that this effect is allele or tissue specific. And finally but most importantly, the requirement of the PDZ domain binding motif in the establishment of the actomyosin cable or in the effect on adherens junctions has not been tested *in vivo*.

Chapter 3: Asymmetric distribution of Echinoid polarizes the actin cytoskeleton during *Drosophila* dorsal closure

3.1 Abstract

During dorsal closure, an epidermal gap, covered by the amnioserosa, is closed by the convergence of two lateral sheets of epidermis. The dorsal-most cells of the epidermis contact the amnioserosa and exhibit a particular planar polarity with an actomyosin cable assembling at their leading edge. The homophilic binding cell adhesion molecule Echinoid (Ed) is essential for the assembly of this actomyosin cable; embryos that lack Ed fail to assemble an actomyosin cable. Ed is expressed in the epidermis but not in the amnioserosa resulting in the asymmetric distribution of Ed around the cortex of the dorsal-most epidermal cells. In this work, we demonstrate that this asymmetric distribution of Ed confers the dorsal-most epidermal cells with a planar polarity that specifically regulates the dynamics of the actin cytoskeleton. This asymmetric distribution of Ed is essential for the accumulation of the actin regulators RhoGEF2, Diaphanous and Enabled at the leading edge and the reciprocal distribution of the polarity protein Baz in the dorsal-most epidermal cells. Finally, we demonstrate by *in vivo* analysis that the PDZ domain binding motif of Ed is dispensable for this function. Our results thus identify a role for the asymmetric distribution of Ed in the establishment of the planar polarity necessary to trigger the assembly of the actomyosin cable at the leading edge possibly via the local activation of the Rho small GTPase Rho1.

3.2 Introduction

The local control of actin dynamics provides the tension necessary for cytokinesis, single cell migration and for the coordinated movements of tissue during the development of multicellular organisms. Concerted epithelial sheet movements require the polarized recruitment of cytoskeleton regulators within the cell to ensure the directionality of the migration. Although the subcellular regulation of the cytoskeleton is crucial to development, our current knowledge of tissue morphogenesis provides little information about the signals that polarize the actin cytoskeleton during epithelial movements *in vivo*.

During *Drosophila* embryogenesis, an eye-shaped epidermal gap, covered by the amnioserosa is closed by the coordinated convergence of two lateral sheets of epidermis (Jacinto et al., 2002). The dorsal-most epidermal cells are specialized cells that exhibit particular actin structures, namely a contractile actomyosin cable and filopodia extensions both assembling at their leading edge, the face of the cells that contacts the amnioserosa (Jacinto et al., 2000; Young et al., 1993). The assembly of the actomyosin cable requires the local recruitment of F-actin and myosin II to the leading edge. Dots of F-actin first appear at actin-nucleating centers (ANCs) located along the leading edge at tricellular junctions. Actin filaments later extend from each ANC along the face of the leading edge (Kaltschmidt et al., 2002). Consistent with this observation, the formin Diaphanous (Dia) is enriched at ANCs and is therefore thought to nucleate and elongate the unbranched F-actin of the actomyosin cable (Homem and Peifer, 2008).

A number of important issues remain unsettled. The Wingless (Wg) signaling pathway initiates the planar polarization during dorsal closure and is essential for the polarized reorganization of the microtubule network, the actin cytoskeleton and the redistribution of junctional complexes around the cortex of the dorsal-most epidermal cells (Kaltschmidt et al., 2002). Yet, our present understanding of actomyosin cable formation lacks a cellular mechanism that promotes the planar polarity to the cells of the leading edge.

Here we identify a role for Echinoid (Ed) in the establishment of the planar polarity of the dorsal-most epidermal cells. Ed is an immunoglobulin domain-containing molecule that interacts homophilically in *trans* at neighboring cell contacts (Laplante and Nilson, 2006; Wei et al., 2005). Previous studies of Echinoid (Ed) revealed its role in the assembly of multicellular actomyosin structures at the interface between cells that express Ed and cells that do not express Ed (Laplante and Nilson, 2006; Lin et al., 2007; Wei et al., 2005). Such actomyosin structures are involved in appendage floor closure during *Drosophila* oogenesis (Laplante and Nilson, 2006) and embryonic dorsal closure (Laplante and Nilson, 2006; Lin et al., 2007). In both cases, elimination of Ed leads to the loss of the actomyosin cable causing subsequent defects in morphogenesis (Laplante and Nilson, 2006). The current model of the molecular function of Ed is based on the interaction of its PDZ domain binding motif and PDZ domain containing partners such as the polarity protein Bazooka (Baz) and the adherens junction component Canoe (Cno) (Lecuit, 2005; Wei et al., 2005). These results

prompted the hypothesis that the differential expression of Ed between two neighbouring tissues results in the formation of an actomyosin cable at their interface.

In this work, we investigated how the distribution of Ed within the dorsal-most epidermal cells contributes to the assembly of the actomyosin cable. During dorsal closure, the epidermis expresses Ed and contacts the non-expressing amnioserosa resulting in the asymmetric distribution of Ed in the dorsal-most epidermal cells; Ed is absent from the leading edge where the epidermal cell contacts the amnioserosa. We found that the assembly of the actomyosin cable is compromised when Ed is maintained at the leading edge similarly to embryos that lack both maternal and zygotic copies of *ed* ($ed^{M/Z}$) (Laplante and Nilson, 2006). Furthermore, the asymmetric distribution of Ed regulates the polarized accumulation of actin regulators such as the Rho1 activator RhoGEF2, the actin-nucleating factor Dia and Enabled (Ena) to the leading edge. Moreover, we found that the polarity protein Baz adopts a particular polarized distribution in the dorsal-most epidermal cells during dorsal closure; Baz is lost from the leading edge and enriched at the anterior-posterior borders of the cells. This polarized distribution of Baz during dorsal closure is dependent on the asymmetric distribution of Ed. Finally, we found that the PDZ domain binding motif of Ed is dispensable for the formation of the actomyosin cable *in vivo*. Our results provide a mechanism by which the asymmetric localization of Ed as a planar polarity signal essential for the local accumulation of actin regulators and result in the assembly of the actomyosin cable at the leading edge.

3.3 Methods and materials

3.3.1 *Drosophila* stains

Germline clones were generated according to our previous work using *w*; ed^{F72}, FRT^{40A} (Laplante and Nilson, 2006). *w*; ed^{F72}, FRT^{40A}, UAS-Ed-ΔP and *w*; ed^{F72}, FRT^{40A}, UAS-Ed-Full were generated by meiotic recombination. For ectopic expression of Ed transgenes: C381 amnioserosa Gal4 driver, paired-Gal4, UAS-Ed-Full, UAS-Ed-ΔP and UAS-Ed-ΔC. For MARCM clones: *y w* hsFlp, UAS-GFP; Tub-Gal80, FRT^{40A}; Tub-Gal4/TM6 (gift of David Hipfner) were crossed to *w*; ed^{F72}, FRT^{40A}, UAS-Ed-ΔP/CyO or *w*; ed^{F72}, FRT^{40A}, UAS-Ed-ΔP/CyO. Pupae were heat shocked for one hour on three consecutive days and the resulting progeny were aged for 6 days and well fed before dissection.

3.3.2 Generation of transgenes

Transgenes were generated by PCR amplification from cDNA RE66591 (DGRC) and inserted in the pENTRY vector (Invitrogen). The resulting clones were sequenced (Génome Québec Innovation Center) and then recombined into the destination vector pTWG (for Ed-ΔC and Ed-ΔP) or pTWH (for Ed-Full) (DGRC). Forward primer for all constructs: 5'-CACCCGTGTGTGCGAACAACAACTCAG-3'. Reverse primers for Ed-Full: 5'-CTAGACAATAATCTCGCGTATG-3'. Reverse primers for Ed-ΔP: 5'-GCGTATGACGCGACGGTTTCTGGC-3'. Reverse primers for Ed-ΔC: 5'-GCTCTTCTTCGATTGATTGCGCTT-3'. There are 9 amino acids left of the cytoplasmic tail of Ed in Ed-ΔC protein.

3.3.3 Generation of Ed antiserum

The cDNA RE66591 (*Drosophila* Genome Resources Center) was used as template to amplify by PCR the fragment encoding the C-terminal domain of Ed, which was then cloned in frame into the pGEX2T-His_6 vector (gift of S. Gunderson). After expression in *E. coli* BL21 cells, the recombinant protein was purified by selection for the His_6 tag and used to immunize rabbits.

3.3.4 Immunohistochemistry

Fixation and staining of ovaries and embryos was performed as described previously (Van Buskirk and Schupbach, 2002; Wieschaus, 1986). For F-actin and pMLC stainings, embryos were fixed in 8% formaldehyde diluted in PBS with 0.5 U/ml phalloidin in heptane for 30 minutes, hand devitellinized and stained for 4 hours with 0.5 U/ml Alexa fluor 555-conjugated phalloidin (dried of Methanol; Molecular Probes). Antibody used were anti-Arm N2 7A1 supernatant (1:100; DSHB), anti-Ena 5G2 supernatant (1:200; DSHB), phospho-myosin II light chain (Thr18 Ser19) (embryo staining, 1:250; Cell Signaling), phosphorylated-myosin II light chain (Ser19) (egg chamber staining, 1:250; Cell Signaling), anti-Zipper (1:600) (gift of D. Kiehart, Young et al., 1993), anti-Dia (1:2500) (gift of S. Wasserman, Afshar et al., 2000), anti-Ed rat (1:1000 Laplante and Nilson, 2006), anti-Ed rabbit (1:1000), anti-Cno (1:500, gift of D. Yamamoto Matsuo et al., 1999), anti-Dlg 4F3 supernatant (1:100; DSHB) anti-Coracle (1:500; gift of R. Fehon), anti-αTubulin (1:100; Sigma), anti-Fmi #74 supernatant (1:50;

DSHB), anti-RhoGEF2 (1:2000) (gift of S. Rogers, Rogers et al., 2004). All secondary antibodies (Molecular Probes) were highly cross-adsorbed Alexa fluor conjugated anti-IgG pre-blocked against fixed embryos and used at a final concentration of 1:1,000 overnight at 4°C.

3.3.5 Microscopy and image analysis

All the images were taken on a Zeiss LSM confocal microscope (McGill CIAN facility). The images were analyzed and cell measurements were performed using the imaging software Volocity (Improvision).

3.4 Results

3.4.1 Expression of Ed transgenes in the amnioserosa is sufficient to maintain endogenous Ed at the leading edge during dorsal closure

From cellularization until stage 11 of embryogenesis, Ed is detectable uniformly in all epidermal and amnioserosa cells (Figure 3.1A, B). During stage 11, the levels of detectable Ed decrease in the amnioserosa and by stage 13, Ed is undetectable in the amnioserosa creating a difference in expression of Ed between the amnioserosa cells and the Ed expressing epidermal cells (Figure 3.1C, E, E') (Laplante and Nilson, 2006). At that stage, the distribution of Ed in the dorsal-most epidermal cells becomes polarized with respect to the leading edge; Ed localizes to the membrane that contacts other Ed expressing cells but is absent at the leading edge where the epidermal cell contacts the Ed non-expressing amnioserosa cells (Figure 3.1E', arrow). Therefore, the

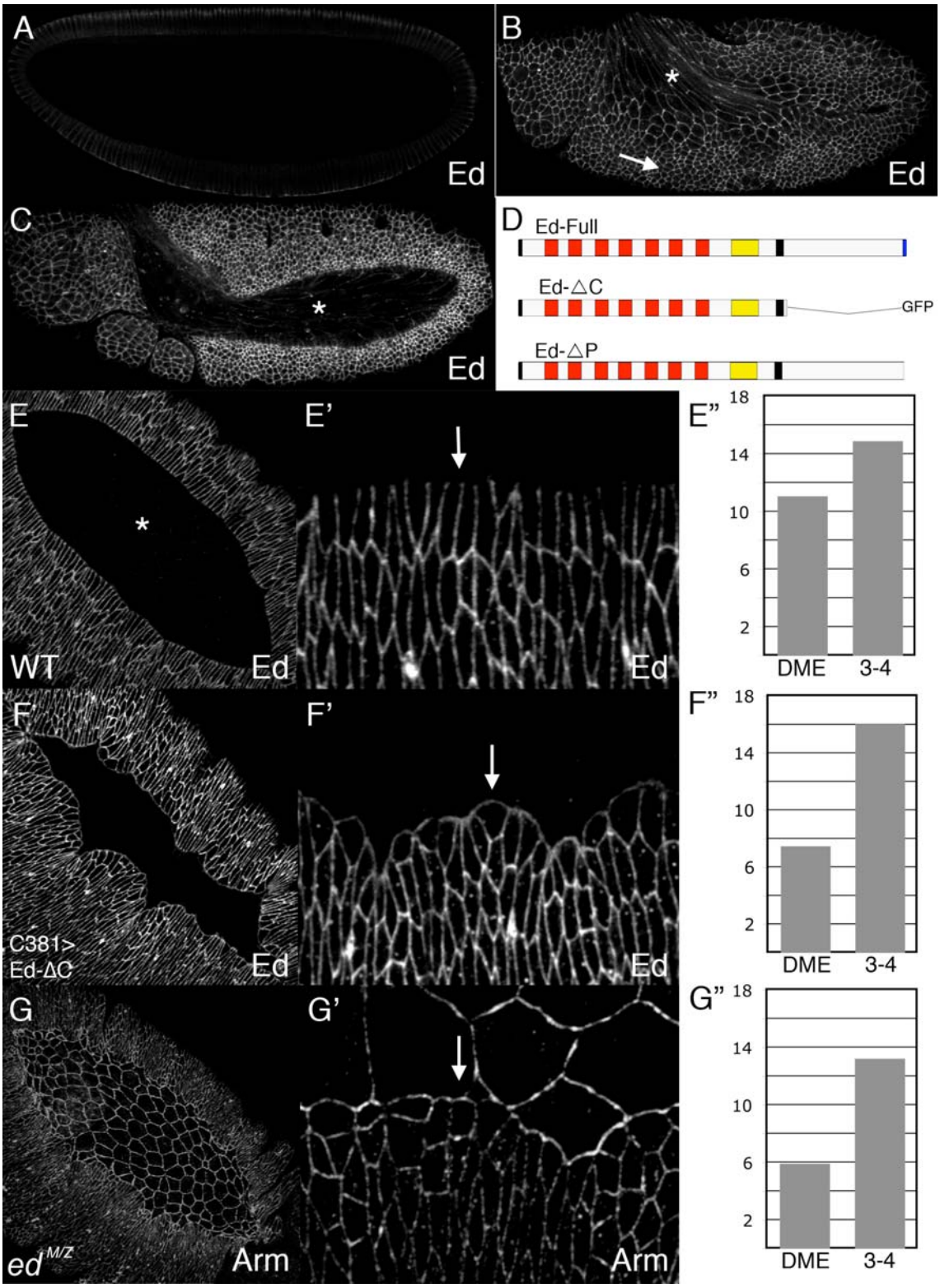
difference in expression of Ed between the amnioserosa and the epidermis results in the asymmetric distribution of Ed in the dorsal-most epidermal cells. Since Ed is a homophilic-binding molecule, we interpret the loss of Ed from the leading edge as a result of the removal of Ed from the neighboring amnioserosa cells (Laplante and Nilson, 2006; Wei et al., 2005). Our previous work showed that embryos mutant for both the maternal and zygotic contributions of *ed* ($ed^{M/Z}$) fail to assemble an actomyosin cable at the leading edge. As the juxtaposition of cells with and without Ed is sufficient to trigger the assembly of an actomyosin cable these results prompted the hypothesis that the differential expression of Ed between the amnioserosa and the epidermis triggered the assembly of the actomyosin cable (Laplante and Nilson, 2006).

Our goal here was to determine whether the asymmetric distribution of Ed in the dorsal-most epidermal cells is essential to the formation of the actomyosin cable at the leading edge. We therefore asked whether endogenous Ed could be maintained at the leading edge during dorsal closure by providing homophilic interaction via the expression of Ed transgenes in the amnioserosa. To maintain endogenous Ed at the leading edge, we used the UAS/Gal4 system for the tissue specific ectopic expression of a transgene that codes the full-length protein (Ed-Full) in the amnioserosa using the amnioserosa driver C381. We hypothesized that the extracellular domain of Ed-Full from the amnioserosa and that of endogenous Ed from the epidermis would interact homophilically in *trans* thus forcing endogenous Ed to be retained at the leading edge of the epidermis during dorsal closure (Figure 3.1D). We chose not to tag the full-length Ed transgene to avoid altering the properties of the PDZ domain binding motif, which

can result in the inability to bind to PDZ-containing protein partners. Ectopic expression of Ed-Full in the amnioserosa during stage 13 results in the maintenance of Ed at the leading edge of the dorsal-most epidermal cells. (Figure S3.1A, A'). Interestingly however, later during dorsal closure, the first row or two of amnioserosa cells, the peripheral amnioserosa cells, are particularly resilient to the ectopic expression of the Ed-Full transgene (Figure S3.1B, B'). We find that this effect is not specific to the genomic insertion site of the transgene as it was observed with all the tested insertion lines (n=4). The lack of Ed-Full in the peripheral amnioserosa cells suggests that those cells downregulate Ed mRNA or Ed protein itself via an unknown mechanism.

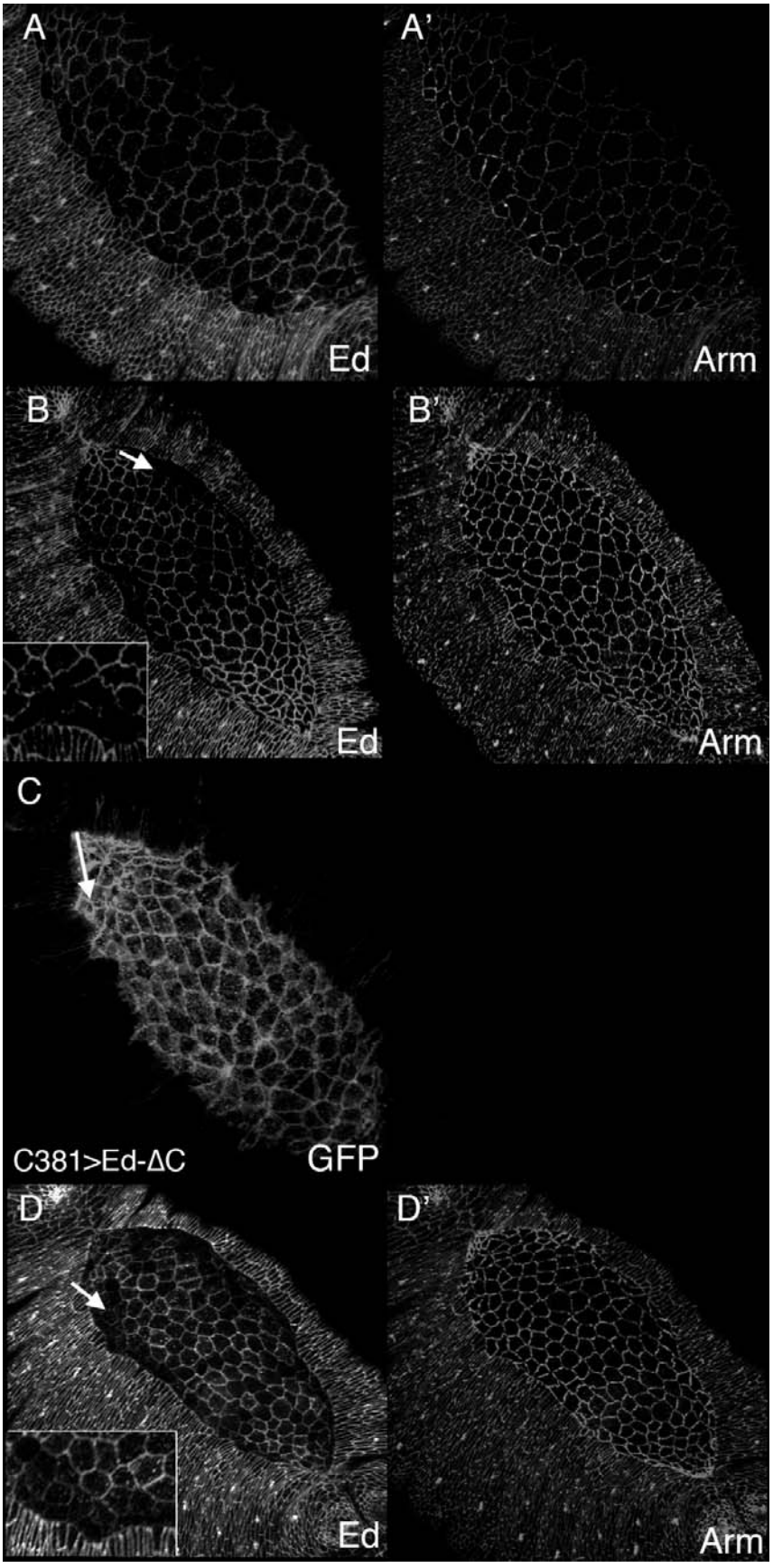
Figure 3.1. Asymmertic distribution of Ed within the dorsal most epidermal cells is abrogated by ectopic expression of Ed in the amnioserosa.

A. Wild type embryo during cellularization stained for Ed. Ed is restricted to the apical portion of the lateral domain of each cell. **B.** Wild type stage-8 embryo stained for Ed. Ed is detectable in all epidermal (arrow) and amnioserosa (asterisk) cells. **C.** Wild type stage-11 embryo stained for Ed. Detectable Ed levels are decreased in the amnioserosa (asterisks). **D.** Schematic representation of the Ed-Full, Ed-ΔC and Ed-ΔP transgenes. Signal sequence (black, left), Immunoglobulin domains (red), Fibronectin type 3 domain (yellow), transmembrane domain (black, center), PDZ domain binding motif (blue). **E, E'.** Wild type embryos stage-13 stained for Ed. Ed is absent from the amnioserosa (asterisk) and from the leading edge of the dorsal most epidermal cells (E', arrow points to the leading edge). **E".** Graph of cell length in wild type embryos during zippering. Maximal dorsal-ventral length dorsal-most epidermal cells (DME; 11.1μm ± 2.7, n=46) and cells in either the 3rd or 4th row (3-4; 14.9μm ± 2.6, n=41). **F, F'.** Wild type embryos expressing Ed-ΔC in the amnioserosa and stained for Ed. Endogenous Ed is maintained at the leading edge and the leading edge remains scalloped during dorsal closure (F'; arrow points to the leading edge). **F".** Graph of cell length in wild type embryos expressing Ed-ΔC in the amnioserosa during zippering. Maximal dorsal-ventral length dorsal-most epidermal cells (DME; 7.5μm ± 2.2, n=77) and cells in either the 3rd or 4th row (3-4; 16.1μm ± 3.1, n=45). **G, G'.** *ed*$^{M/Z}$ embryos stained for Arm. The leading edge remains scalloped during dorsal closure (G'; arrow points to the leading edge). **G".** Graph of cell length in *ed*$^{M/Z}$ embryos during zippering. Maximal dorsal-ventral length dorsal-most epidermal cells (DME; 5.9μm ± 1.5, n=59) and cells in either the 3rd or 4th row (3-4; 13.22μm ± 1.9, n=66).


A
Ed
B
*
Ed
C
*
Ed
D
Ed-Full
Ed-△C
GFP
Ed-△P
E
*
WT
Ed
E'
Ed
E"
18
14
10
6
2
DME
3-4
F'
C381>
Ed-ΔC
Ed
F'
Ed
F"
18
14
10
6
2
DME
3-4
G
ed M/Z
Arm
G'
Arm
G"
18
14
10
6
2
DME
3-4

Supplemental Figure 3.1. Expression of Ed-Full and Ed-ΔP is variable in the peripheral amnioserosa cells.

A-B'. Wild type embryos expressing FullEd in the amnioserosa and stained for Ed (A, B) and Arm (A', B'). The transgene becomes undetectable in the first row of amnioserosa cells in later stages of dorsal closure (B; arrow). **C.** Wild type embryo expressing Ed-ΔC in the amnioserosa stained for GFP. Ed-ΔC is expressed throughout the amnioserosa, arrow points to the peripheral amnioserosa cells. **D, D'.** Wild type embryos expressing Ed-ΔP in the amnioserosa and stained for Ed (D) and Arm (D'). The transgene becomes undetectable in the first row of amnioserosa cells in later stages of dorsal closure (D; arrow).


A
Ed
A'
Arm
B
Ed
B'
Arm
C
C381>Ed-ΔC
GFP
D
Ed
D'
Arm

We then hypothesized that the extracellular domain of Ed might be sufficient to bind to and stabilize endogenous Ed at the leading edge. We therefore expressed an Ed transgene that lacks the cytoplasmic tail (Ed-ΔC) in the amnioserosa and assessed whether it can maintain endogenous Ed at the leading edge of the epidermis (Figure 3.1D). This construct cannot be recognized with our antibody, which was raised against the cytoplasmic tail of Ed, and was tagged with a green fluorescent protein (GFP) at the carboxy-terminus thereby allowing us to distinguish endogenous Ed from the Ed-ΔC. When Ed-ΔC is expressed in the amnioserosa, endogenous Ed is detectable at the leading edge of the dorsal-most epidermal cells (Figure 3.1F, F'). This observation suggested that the extracellular domain of Ed-ΔC expressed by the amnioserosa cells is indeed sufficient to interact homophilically with the extracellular domain of endogenous Ed (n=5 different transgenic insertions tested). Unlike Ed-Full, Ed-ΔC is detectable uniformly in the amnioserosa cells throughout dorsal closure (Figure S3.1C). This system was used in subsequent experiments to maintain endogenous Ed uniformly distributed around the cortex of the dorsal-most epidermal cells and was referred to as the Ed-LE embryo sample.

The appearance of the leading edge in Ed-LE embryos is strikingly similar to that of $ed^{M/Z}$ embryos. Indeed, in both genetic backgrounds embryos exhibit a jagged leading edge (Figure 3.1, compare F, F' to G,G') different from the smooth straight leading edge of wild type embryos (Figure 3.1E, E'). Indeed, in both $ed^{M/Z}$ and Ed-LE embryos, the dorsal-most epidermal cells remain short while they normally elongate along the dorsal-ventral axis (Figure 3.1E', F', G'). The tension provided by the

actomyosin cable in the dorsal-most epidermal cells is thought to cause their elongation along the dorsal-ventral axis (Jacinto et al., 2002). We measured the length of dorsal-most epidermal cells in a wild type embryo (measured along the dorsal-ventral axis) and found that on average a cell measures 11.1μm±2.7 (n=46) (Figure 3.1E"). However, the maximal length of a dorsal-most epidermal cell in Ed-LE embryos is 7.5μm±2.2 (n=77) and 5.9μm±1.5 (n=59) in $ed^{M/Z}$ embryos (Figure 3.1F", G"). Furthermore, the JNK and Decapentaplegic (Dpp) pathways are responsible for the elongation of the subsequent rows of epidermal cells independently of the actomyosin cable (Ricos et al., 1999; Riesgo-Escovar and Hafen, 1997). Therefore, it is worth noting that the subsequent rows of epidermal cells located more ventrally do elongate in $ed^{M/Z}$ and Ed-LE embryos similarly to wild type embryos. The maximal length of a 3rd or 4th row epidermal cell in a wild type embryo is 14.9μm±2.6 (n=41) (Figure 3.1E"). Similarly, the maximal length of a dorsal-most epidermal cell in Ed-LE embryo is 16.1μm±3.1 (n=45) and 13.2μm±1.9 (n=66) in $ed^{M/Z}$ embryo (Figure 3.1F", G"). As the elongation of the first row of epidermal cells and the shape of the leading edge has been linked to the tension generated by the contractile actomyosin cable, the appearance of the leading edge in $ed^{M/Z}$ and Ed-LE embryos suggests that the actomyosin cable is absent in those embryos.

3.4.2 The PDZ domain binding motif is dispensable for the downregulation of the Ed transgene in the peripheral amnioserosa cells

The pattern of ectopic Ed-Full in the amnioserosa suggests the existence of a mechanism that prevents the expression of Ed in the peripheral amnioserosa cells during dorsal closure. Since Ed-ΔC can circumvent this regulation, we tested whether this

mechanism relies on the presence of the PDZ domain binding motif in the cytoplasmic tail of Ed. This motif is the only characterized motif of the cytoplasmic tail of Ed and consists of the last 4 amino acids EIIV at the carboxy-terminus. We tested whether a construct that lacks the PDZ domain binding motif (Ed-ΔP) can overcome the downregulation in the amnioserosa, which affects our Ed-Full transgene (Figure 3.1D). We observed that Ed-ΔP is downregulated in the peripheral amnioserosa cells similarly to the Ed-Full construct (Figure S3.1D, D'). This effect was detected with all the insertion lines tested and is therefore unlikely to be due to the genomic insertion site (n=7). The inability to express Ed-Full and Ed-ΔP but not Ed-ΔC in the first rows of amnioserosa cells throughout dorsal closure raises the possibility that the amnioserosa cells recognize a sequence in the cytoplasmic tail of Ed, independent of the PDZ domain binding motif, and rid those cells of Ed.

3.4.3 Asymmetric Ed distribution is essential for the assembly of the actomyosin structure at the leading edge

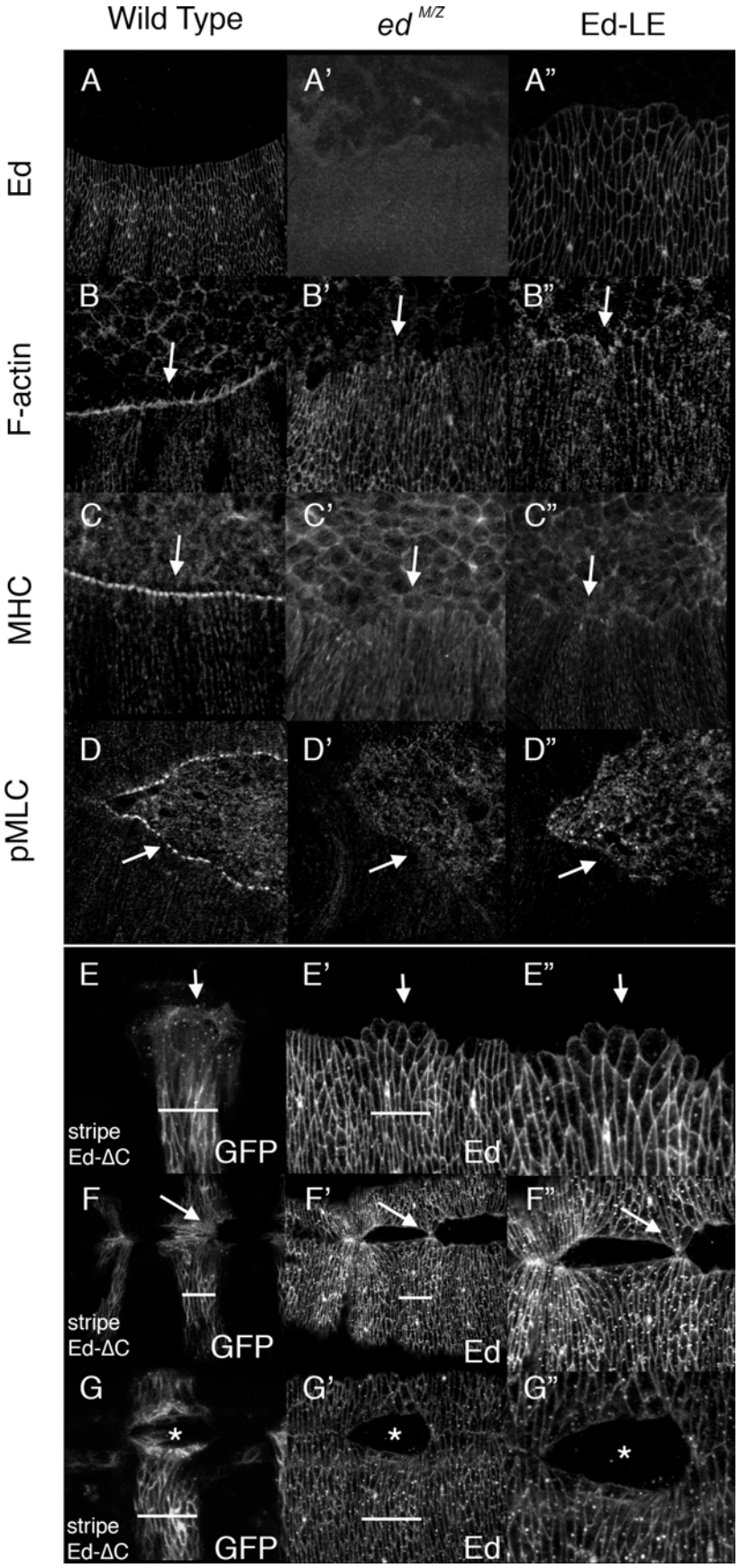
Disrupting the asymmetric distribution of Ed in the dorsal-most epidermal cell either by removing Ed completely in $ed^{M/Z}$ embryos or by maintaining Ed uniformly distributed around the cell cortex in Ed-LE embryos results in failure in cell elongation and a jagged leading edge. These observations prompted the hypothesis that the asymmetric distribution of Ed in the dorsal-most epidermal cells is essential to the production of the actomyosin cable. To test this hypothesis, we compared the cytoskeletal changes that occur in the dorsal-most epidermal cells during dorsal closure between wild type embryos that exhibit asymmetric distribution of Ed, $ed^{M/Z}$ embryos

and Ed-LE embryos (Figure 3.2A-A"). We used $ed^{M/Z}$ and Ed-LE embryos as two systems that exhibit a symmetrical distribution of Ed around the dorsal-most epidermal cells to distinguish between the consequences of the complete lack of Ed in $ed^{M/Z}$ embryos and the role of the asymmetric distribution of Ed in the dorsal-most epidermal cells.

During the formation of the actomyosin cable, F-actin polymerization is first initiated at actin-nucleating centers (ANCs) located along the leading edge at tricellular junctions where epidermal and amnioserosa cells contact (Kaltschmidt et al., 2002). It is thought that F-actin nucleated from the ANCs is then re-organized into anti-parallel bundles along the leading edge to shape the backbone of the multicellular actomyosin cable (Figure 3.2B). In $ed^{M/Z}$ embryos however, F-actin fails to accumulate at the leading edge; the intensity of F-actin appears uniform around the dorsal-most epidermal cells (Figure 3.2B') (Lin et al., 2007). Strikingly, F-actin also fails to accumulate at the leading edge of Ed-LE embryos similarly to $ed^{M/Z}$ embryos (Figure 3.2B"). In wild type embryos, filopodia protruding from the leading edge can be successfully preserved during fixation (Figure 3.2B) (Kaltschmidt et al., 2002). Interestingly, $ed^{M/Z}$ and Ed-LE embryos rarely display filopodia extending from the leading edge suggesting a role for the asymmetric distribution of Ed in the formation of filopodia (Figure 3.2B', B" Lin et al., 2007).

Figure 3.2. Differential Ed localization within the dorsal-most epidermal cells is essential for the assembly of the actomyosin structure at the leading edge.

A, B, C, D. Wild type embryos. **A', B', C', D'**. *ed*$^{M/Z}$ mutant embryos. **A", B", C", D".** Wild type embryos ectopically expressing Ed-ΔC in the amnioserosa. **A-A".** Ed. A'. The background of the staining was enhanced to show the presence of tissue in the absence of Ed. **B-B".** F-actin. **C-C".** MHC. **D-D".** pMLC. **E-E".** Embryo expressing Ed-ΔC in paired-Gal4 positive stripes (bar). **E.** Ed-ΔC (GFP). **E', E".** Ed. **F-F".** Embryo stage 15 expressing Ed-ΔC in paired-Gal4 positive stripes (bar). Ed-ΔC expressing cells make premature contact (arrow). **F.** Ed-ΔC (GFP). **F', F".** Ed**. G-G".** Embryo stage 17 expressing Ed-ΔC in paired-Gal4 positive stripes (bar). A hole remains at the dorsal midline (asterisk). **F.** Ed-ΔC (GFP). **F'-F".** Ed.


Wild Type
ed M/Z
Ed-LE
Ed
F-actin
MHC
pMLC
A
A'
A"
B
B'
B"
C
C'
C"
D
D'
D"
E
E'
E"
F
F'
F"
G
G'
G"
stripe Ed-ΔC
GFP
Ed
*

In wild type embryos, the presence of the actomyosin cable is also highlighted by the conspicuous accumulation of myosin II heavy chain (MHC; encoded by *zipper*) (Figure 3.2C). In contrast, MHC is not detectable at the leading edge of $ed^{M/Z}$ mutant embryos indicating that the actomyosin cable cannot assemble in the complete absence of Ed (Figure 3.2C', Laplante and Nilson, 2006). Similarly, the accumulation of MHC is compromised at the leading edge of Ed-LE embryos (Figure 3.2C"). A similar phenotype is observed when embryos are stained for the active phosphorylated form of the myosin II regulatory light chain (pMLC) (Figure 3.2D-D"). The lack of F-actin and myosin II accumulation in $ed^{M/Z}$ and Ed-LE embryos suggests that not only is Ed absolutely essential for the formation of the actomyosin cable but its distribution around the dorsal-most epidermal cell needs to be asymmetric; Ed present at the contact with Ed expressing epidermal cells and absent from the contact with the Ed non-expressing amnioserosa.

The Ed-LE embryos provide an ideal system to study the role of the actomyosin cable during dorsal closure. We generated embryos that express Ed-ΔC in paired segmental stripes. The paired driver is expressed around the entire circumference of the embryo including both epidermal and amnioserosa cells. Such embryos therefore exhibit stripes of dorsal-most epidermal cells with uniform Ed distributed around their cortex (Ed-LE stripe) alternating with wild type cells in which Ed is asymmetrically distributed. This arrangement allows for side-by-side comparison between cells that exhibit an actomyosin cable and cells that don't. In such embryos, the cells of the Ed-LE stripe adopt a fan shape with their leading edge splayed wide along the anterior-

posterior axis compared to the narrow leading edge of the wild type cells. Moreover, the cells of the Ed-LE stripe acquire a migration advantage over the neighbouring wild type cells that may allow them to contact the contralateral sheets of epidermis prematurely (n=37) (Figure 3.2E-F"). These observations are consistent with the hypothesis that the actomyosin cable maintains tension in the leading edge and thus restrains the epidermal migration during closure (Kiehart et al., 2000). Finally, at stage 17, such embryos often display a dorsal hole (3/4 embryos) that coincides with the position of the central Ed-LE stripe suggesting that although cells without an actomyosin cable gain a migrational advantage, their final suture is possibly defective (Figure 3.2G-G").

3.4.4 The asymmetric distribution of Ed is required to establish the planar polarized actin regulating machinery in the cells of the leading edge

Our data so far indicate that the local assembly of the actomyosin cable at the leading edge requires the asymmetric distribution of Ed within the dorsal-most epidermal cells. It is sensible to posit that the polarized accumulation of actin regulators also depends on the asymmetric distribution of Ed. The assembly of the actomyosin cable requires signaling from the Rho small GTPase Rho1 (Harden et al., 1999; Magie et al., 1999; Wood et al., 2002). Like other Rho small GTPases, Rho1 alternates between an active GTP-bound state and an inactive GDP-bound state. The activity of Rho small GTPases is tightly controlled within cells by regulatory molecules that influence the nature of the nucleotide bound to the protein. Guanine nucleotide exchange factors (GEFs) exchange GDP for GTP hence activating Rho small GTPases

while GTPase activating proteins (GAPs) promote the hydrolysis of GTP to GDP thus inactivating the protein (Van Aelst and D'Souza-Schorey, 1997) (Van Aelst and Symons, 2002). Since the actomyosin cable assembles only at one face of the dorsal-most epidermal cell, the leading edge, we hypothesized that Rho1 is locally activated at the leading edge under wild type conditions.

RhoGEF2, one of the GEFs known to activate Rho1 in *Drosophila*, is essential for the organization of the apical actin cytoskeleton and for the stabilization and activation of myosin II during ventral furrow invagination (Fox and Peifer, 2007). We therefore hypothesized that RhoGEF2 could regulate Rho1 during dorsal closure and investigated its localization in wild type embryos during the different stages of dorsal closure. Wild type embryos exhibit a conspicuous enrichment of RhoGEF2 at the leading edge during dorsal closure at the stages when the actomyosin cable is present (Figure 3.3A). This localization profile of RhoGEF2 during dorsal closure is therefore consistent with the possible local activation of Rho1 at the leading edge. We then examined the localization of RhoGEF2 in $ed^{M/Z}$ mutant embryos and found that there is no obvious accumulation of the protein at the leading edge throughout dorsal closure (Figure 3.3A'). Finally, the accumulation of RhoGEF2 at the leading edge is abrogated in Ed-LE dorsal-most epidermal cells (Figure 3.3A"). Our results show that the polarized distribution of Ed within the dorsal-most epidermal cells is necessary for the polarized accumulation of RhoGEF2 at the leading edge. We further interpret these results to propose that the asymmetric distribution of Ed activates Rho1 at the leading

edge, which in turn activates the machinery to generate an actomyosin cable (Figure 3.3E-E").

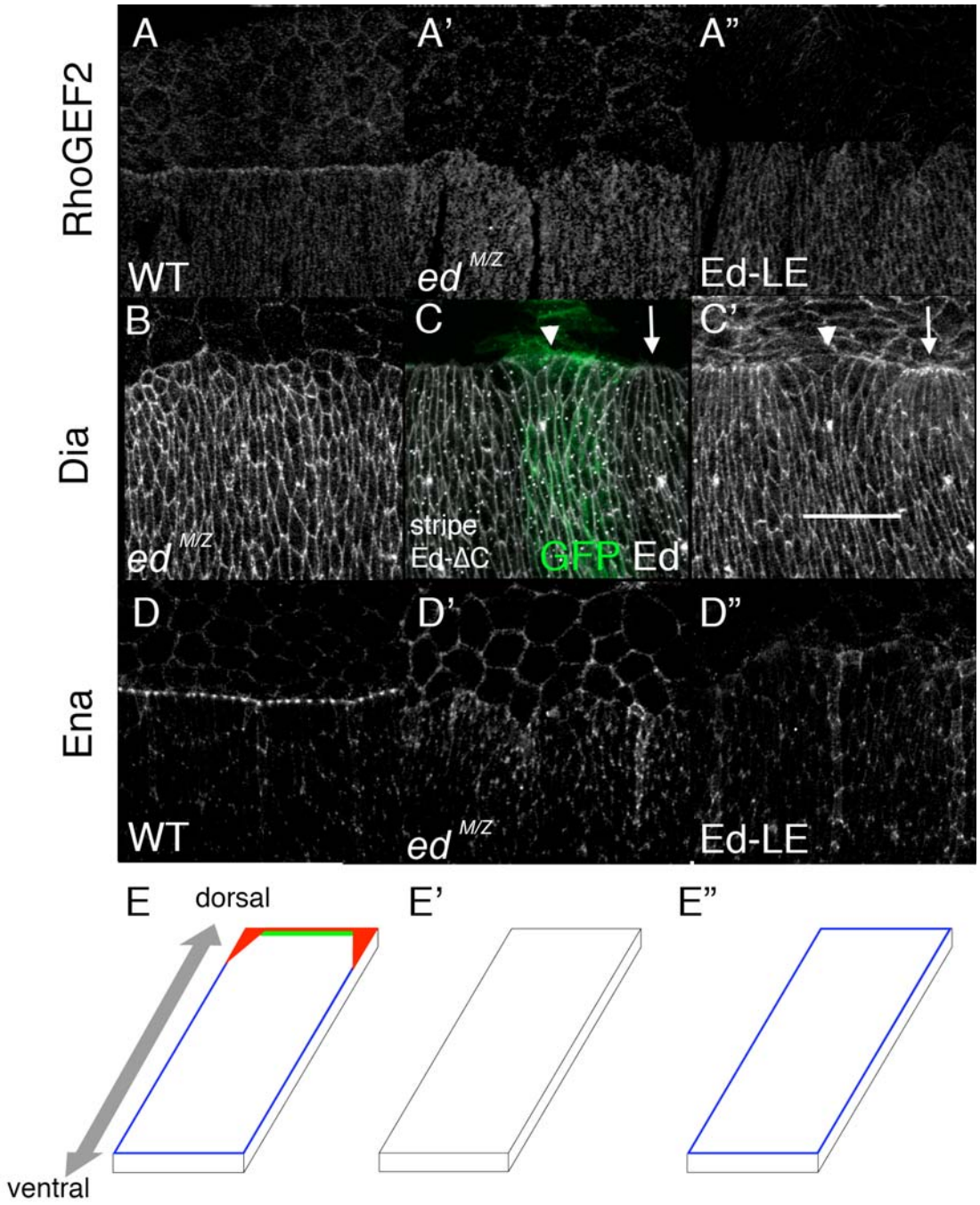
The Diaphanous-type of formins nucleate and promote the elongation of unbranched actin filaments, which participate in the formation of actomyosin contractile structures such as the cytokinetic ring (Pollard, 2007). Rho small GTPase regulates the activity of Dia; active Rho1-GTP binds to Dia thus relieving an inhibitory intramolecular interaction and allowing Dia to homo-dimerize and perform its actin related function (Watanabe et al., 1997). Dia was recently shown to accumulate at ANC during the sweeping phase of dorsal closure consistent with its function in polymerizing F-actin (Figure 3.3C, C'Homem and Peifer, 2008). As Dia is essential for cytokinesis, its direct role in the assembly of the actomyosin cable during dorsal closure cannot be assessed directly by analysis of $dia^{M/Z}$ embryos but can be extrapolated from its characterized functions in other systems (Grosshans et al., 2005). It is therefore reasonable to speculate that the enriched Dia at the ANC is responsible for the polymerization of the unbranched F-actin that creates the backbone of the actomyosin cable. Since F-actin fails to accumulate when Ed is either uniformly distributed around the cell cortex or when it is completely missing, we investigated whether the accumulation of Dia at the ANCs is dependent on the asymmetric distribution of Ed in the dorsal-most epidermal cells. In $ed^{M/Z}$ mutant embryos, Dia fails to accumulate at the ANCs along the leading edge (Figure 3.3B). The distribution of Dia was then analyzed in embryos that express Ed-ΔC in epidermal stripes of the *paired* gene expression

pattern. Such embryos display a wild type pattern of Dia enriched at the ANC in the wild type cells. In contrast, Dia fails to accumulate to the ANC in the cells of the Ed-LE stripe where Ed is symmetrically distributed around the cell cortex (Figure 3.3C, C' arrowheads). Thus, the enrichment of the Formin Dia at the ANC is dependent on the asymmetric distribution of Ed in the cells of the leading edge (Figure 3.3E-E").

The actin regulator Enabled/VASP (Ena) becomes strongly enriched at the ANCs and is thought to prevent the barbed end of actin filaments from being bound by capping proteins thus promoting the elongation of the filaments during dorsal closure (Figure 3.3D Gates et al., 2007). This work has also shown that Ena is essential to the formation of filopodia from the leading edge but its loss doesn't influence the assembly of the F-actin belt. We investigated the polarized distribution of Ena in $ed^{M/Z}$ and Ed-LE embryos because such embryos exhibit few filopodia. Prior to dorsal closure, the distribution of Ena in both $ed^{M/Z}$ and Ed-LE embryos is indistinguishable to that of wild type embryos (data not shown). During dorsal closure however, Ena fails to accumulate at the ANC in both $ed^{M/Z}$ and Ed-LE embryos and the weak localization of Ena at tricellular junctions is maintained throughout dorsal closure (Figure 3.3D', D"). Therefore, the asymmetric distribution of Ed within the dorsal-most epidermal cells is essential for the polarized accumulation of Ena during dorsal closure. Furthermore, since Ena is required for the proper formation of filopodia at the leading edge (Gates et al., 2007), our result proposes that the lack of filopodia extensions in $ed^{M/Z}$ and Ed-LE embryos may be caused by the poor accumulation of Ena at the leading edge.

Figure 3.3. The asymmetric distribution of Ed is required to establish the planar polarized actin regulating machinery in the cells of the leading edge.

A, B, D. Wild type embryos. **A', D'.** *ed*$^{M/Z}$ embryo. **A", D".** Ed-LE embryos. **C, C".** Embryo that expresses Ed-ΔC in a *paired*-Gal4 stripe generating a stripe of Ed-LE flanked by wild type cells. **A-A".** RhoGEF2. **B, C, C'.** Dia. C,C'. Dia is enriched at the ANCs in wild type cells (arrows) whereas it is not enriched at the leading edge of the cells in the Ed-LE stripe (arrowheads). **D-D".** Ena. **E-E".** Schematic representation of dorsal-most epidermal cells in wild type embryo (E), *ed*$^{M/Z}$ embryo (E') and Ed-LE embryo (E"). ANC (enriched in F-actin, Dia, Ena) are represented as red triangles at the tricellular junctions of the leading edge. The F-actin cable is represented as the red line at the leading edge and myosin II and RhoGEF2 as the green line. The distribution of Ed around the cell cortex is depicted as a blue line.


RhoGEF2
A
A'
A"
WT
ed M/Z
Ed-LE
Dia
B
C
C'
ed M/Z
stripe
Ed-ΔC
GFP Ed
Ena
D
D'
D"
WT
ed M/Z
Ed-LE
E
dorsal
E'
E"
ventral

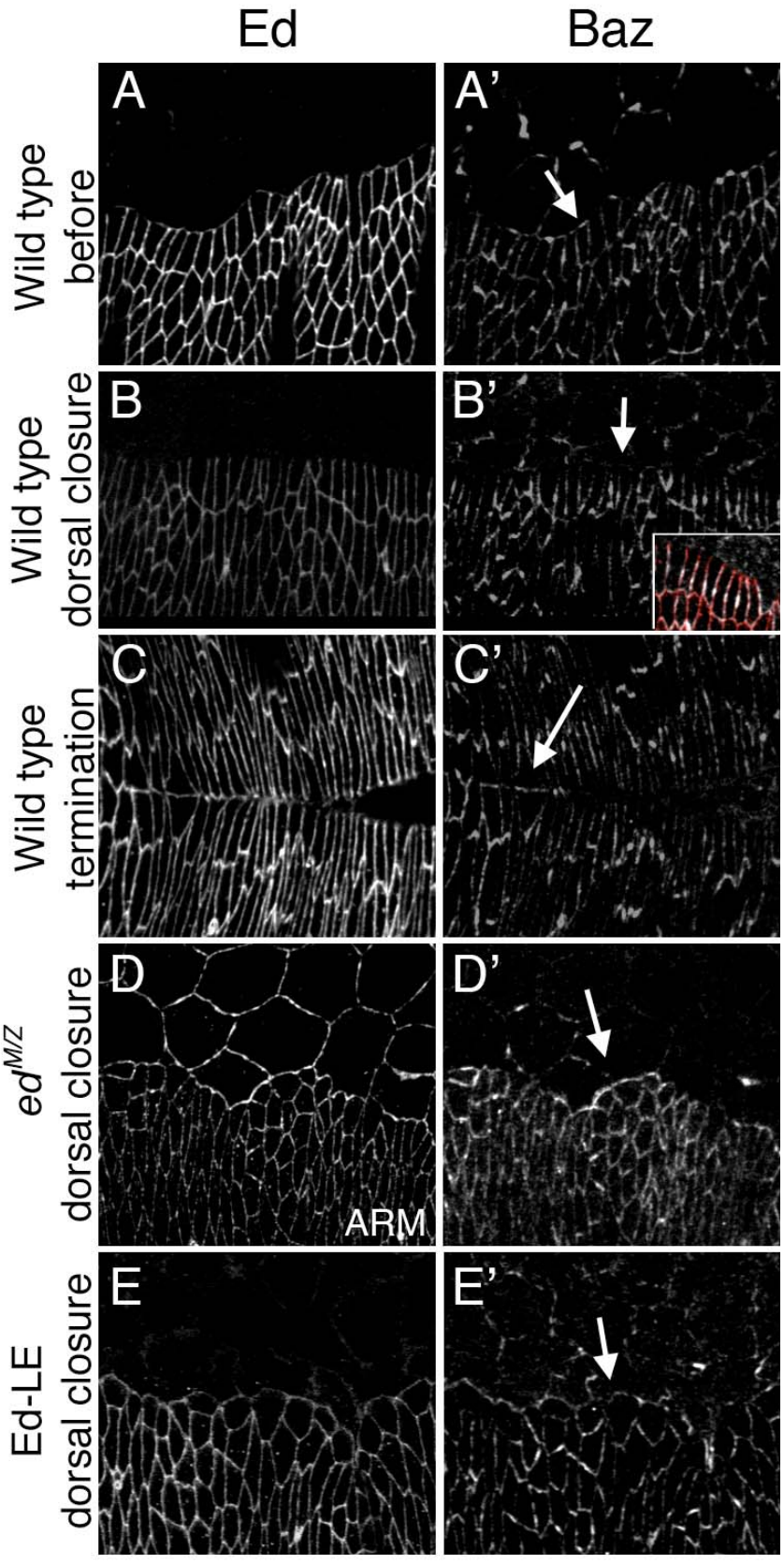
3.4.5 The polarized distribution of Ed in the dorsal-most epidermal cells affects the localization of Bazooka

Our results so far indicate that the asymmetric localization of Ed in the dorsal-most epidermal cells is essential for the polarized accumulation of the actin regulators RhoGEF2, Dia and Ena and the assembly of the actomyosin cable. The planar polarized enrichment of MHC at the leading edge during dorsal closure is reminiscent of its polarized distribution during germ band extension. In the course of that morphogenetic process, epidermal cells intercalate and exchange neighbours by coordinating the shrinkage of anterior-posterior contacts with the expansion of dorsal-ventral cell contacts. The polarity protein Baz, *Drosophila* homologue of Par-3, adopts a polarized distribution in intercalating epidermal cells and becomes enriched at the dorsal-ventral cell borders. Meanwhile, MHC accumulates at the reciprocal anterior-posterior cell borders (Zallen and Wieschaus, 2004). Although it is not clear whether the reciprocal distributions of Baz and MHC are functionally interrelated, we hypothesized that the distribution of Baz may also be polarized in the dorsal-most epidermal cells. Our observations show that in wild type embryos, Baz is detectable at all faces of the dorsal-most epidermal cells prior to dorsal closure (Figure 3.4A, A' arrow). As dorsal closure begins, Baz is gradually lost from the leading edge and accumulates as an aggregate halfway along the anterior-posterior cell borders of the dorsal-most epidermal cells (Figure 3.4B, B' arrow and inset shows aggregates). This interesting re-distribution of Baz coincides with the loss of Ed from the leading edge (Figure 3.4B). During the termination of dorsal closure, when the two lateral sheets of epidermis join at the dorsal

midline, Baz accumulates at the new contacts created between the epidermal cells (Figure 3.4C, C', inset). Our observations thus show that Baz adopts a polarized distribution in the dorsal-most epidermal cells during dorsal closure.

Figure 3.4. The polarized distribution of Ed in the dorsal most epidermal cells influences the localization of Baz.

Arrows point to the leading edge. Embryos stained for Ed (**A,B,C,E**), Arm (**D**) and Baz (**A',B',C',D',E'**). Embryos prior to dorsal closure (**A-A'**), during dorsal closure (**B,B',D,D',E,E')** and at the end of dorsal closure (**C,C'**). **A-C'.** During wild type dorsal closure, Baz is lost from the leading edge of the dorsal most epidermal cells (arrows in A' and B') and aggregate along the anterior-posterior border of the dorsal-most epidermal cells (B', inset; Ed in red and Baz in white). At the end of dorsal closure, when the cells contact at the dorsal midline, Baz comes back at the cell contacts (arrow in C'). **D,D'.** $ed^{M/Z}$ embryos retain Baz at the leading edge during dorsal closure. Arm staining is shown to highlight the outline of the cells (arrow in D'). **E,E'.** Ed-LE embryos maintain Baz at the leading edge during dorsal closure (arrow in E').


Ed
Baz
A
A'
Wild type
before
B
B'
Wild type
dorsal closure
C
C'
Wild type
termination
D
D'
ARM
ed^M/Z
dorsal closure
E
E'
Ed-LE
dorsal closure

We then investigated whether the re-distribution of Baz during dorsal closure is dependent on the distribution of Ed. In both $ed^{M/Z}$ and Ed-LE embryos, Baz is initially distributed uniformly around the cortex of the cells prior to dorsal closure similarly to wild type embryos (data not shown). However, Baz fails to be re-distributed within the dorsal-most epidermal cells during sweeping and remains at the leading edge of the dorsal-most epidermal cells (Figure 3.4D, D'). Likewise, Baz remains uniformly distributed around the dorsal-most epidermal cells in Ed-LE embryos throughout dorsal closure (Figure 3.4E, E'). These results suggest the presence of Ed is essential for the polarized re-distribution of Baz in the dorsal-most epidermal cells and that Ed must be asymmetrically distributed itself to obtain the polarized distribution of Baz during dorsal closure.

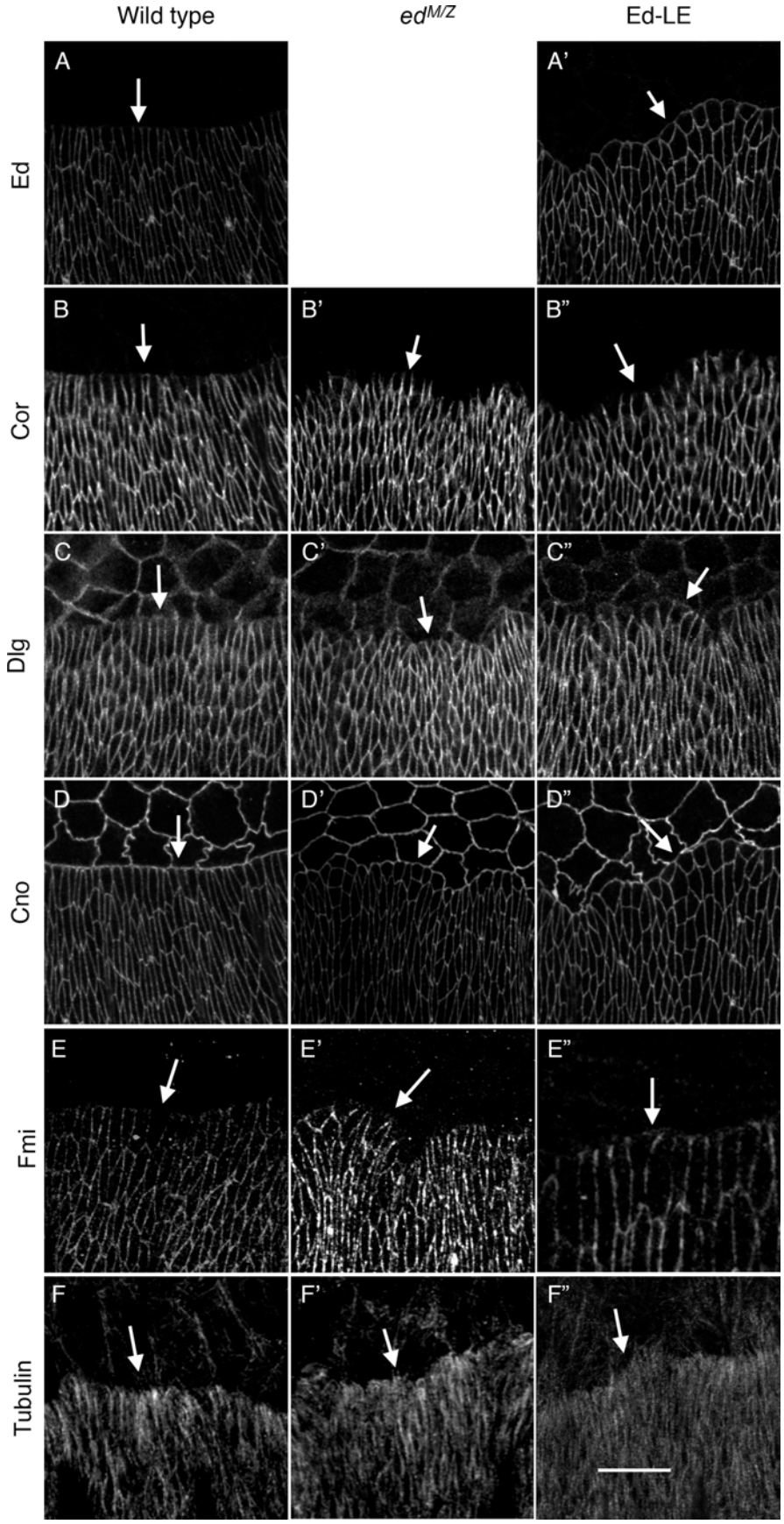
3.4.6 The distribution of Ed does not affect the polarized localization of septate junctions and adherens junctions, Flamingo and the microtubule network.

So far, our results indicate that the asymmetric distribution of Ed in the dorsal-most epidermal cells confers a planar polarity to the cells, which is essential for the localized accumulation of regulators of the actin cytoskeleton and the polarized distribution of Baz. We therefore considered the possibility that the abrogation of the asymmetric polarization of Ed causes the general loss of polarity in the dorsal-most epidermal cells. We investigated whether the distribution of other polarized markers in the dorsal-most epidermal cells was also dependent on the asymmetric distribution of Ed.

The distribution of septate junctions is polarized in the dorsal-most epidermal cells; septate junctions are present at contacts between epidermal cells but are absent at the leading edge where the dorsal-most epidermal cells contact the amnioserosa (Figure 3.5A, B, C) (Kaltschmidt et al., 2002; Magie et al., 1999). Septate junctions only appear at the leading edge when the two sheets of epidermis join together and adhere at the dorsal midline (data not shown; Magie et al., 1999). In *rho1* mutant embryos, Fasciclin III, a component of septate junctions, appears prematurely at the leading edge (Magie et al., 1999). The polarized accumulation of RhoGEF2, Dia and the actomyosin cable at the leading edge suggests that Rho1 is active specifically at the leading edge. We therefore investigated whether the asymmetric localization of Ed influences the polarized distribution of septate junction markers, Coracle (Cor) and Disc Large (Dlg), in $ed^{M/Z}$ and Ed-LE embryos. In $ed^{M/Z}$ embryos, Cor and Dlg are both absent from the leading edge like in wild type embryos (Figure 3.5B', C'). Finally, the localization of Cor and Dlg is unaffected in Ed-LE embryos (Figure 3.5A', B", C"). These results suggest that the polarized localization of the septate junctions at the leading edge is independent of the cortical distribution of Ed. Similarly, we saw no difference in the leading edge distribution of the adherens junction markers Cno and Armadillo between wild type, $ed^{M/Z}$ and Ed-LE embryos (Figure 3.5D-D" and data not shown). These observations suggest that the septate and adherens junctions are not visibly altered by the absence or by changes in the distribution of Ed in the dorsal-most epidermal cells.

Figure 3.5. The distribution of Ed is not responsible for the polarization of septate junctions, adherens junctions, Flamingo and the microtubule network.

Embryos during dorsal closure stage. Arrows point to the leading edge. **A, B, C, D, E, F, G.** Wild type embryos. **B', C', D', E', F', G'.** $ed^{M/Z}$ mutant embryos. **A', B", C", D", E", F", G".** Ed-LE embryos. **A, A'.** Ed. **B-B".** Cor. **C-C".** Dlg. **D-D".** Cno. **E-E".** Arm. **F-F".** Fmi. **G-G".** α-Tubulin. Bar represent a stripe of Ed-LE cells flanked by wild type cells.


Wild type
ed^M/Z
Ed-LE
Ed
Cor
Dlg
Cno
Fmi
Tubulin
A
A'
B
B'
B"
C
C'
C"
D
D'
D"
E
E'
E"
F
F'
F"

Components of the planar polarity core complex are distributed asymmetrically in the dorsal-most epidermal cells prior to the assembly of the actomyosin cable (Kaltschmidt et al., 2002). We investigated whether the distribution of one of the components, the non-classical cadherin Flamingo (Fmi), was influenced by the distribution of Ed. In wild type dorsal-most epidermal cells, Fmi is detectable at all faces of the cell that contact other epidermal cells but is absent from the amnioserosa juxtaposed leading edge (Figure 3.5 E). When the asymmetric distribution of Ed is abrogated in either $ed^{M/Z}$ or Ed-LE embryos, the distribution of Fmi remains polarized along the dorsal-ventral axis (Figure 3.5 E', E"). This observation indicates that the distribution of Fmi is independent of that of Ed in the dorsal-most epidermal cells.

Prior to dorsal closure, the microtubule network appears to be randomly organized in the cytoplasm of the dorsal-most epidermal cells. However, parallel bundles of microtubules orient perpendicular to the leading edge during dorsal closure phase (Figure 3.5F) (Jankovics and Brunner, 2006). We analyzed the distribution of microtubules in $ed^{M/Z}$ and Ed-LE embryos and observed that the polarization of the microtubule network was not visibly altered when the asymmetric distribution of Ed was abrogated (Figure 3.5F', F"). Therefore, the polarization of the microtubule network is independent of the distribution of Ed in the dorsal-most epidermal cells.

The results we have thus far collected indicate that the asymmetric distribution of Ed in the dorsal-most epidermal cells is not sufficient to influence the general polarity of those cells. Rather the distribution of Ed in the dorsal-most epidermal cells dictates

the polarity of specific components namely actin regulators, the actin cytoskeleton and the polarity protein Baz.

3.4.7 Homophilic interaction in *trans* rescues the clone border phenotypes in *ed* mosaic follicular epithelia

Interfaces of differential Ed expression can also be created ectopically by generating mosaic tissues where clones of *ed* mutant cells contact wild type Ed expressing neighbours (Laplante and Nilson, 2006). Under those conditions, the Ed expressing cells located at the clone border fail to localize Ed at the membrane that contacts the Ed non-expressing cell resulting in the asymmetric distribution of Ed in the Ed expressing cells; Ed is present at the faces of the cell that touch other Ed expressing cells but is absent at the membrane that contacts an Ed non-expressing cell.

During dorsal closure, the difference of Ed expression occurs at the interface of two tissues with cells of different fate: the epidermis and the amnioserosa. We therefore used the follicular epithelium as a complementary model tissue in which all the cells are of the same fate to confirm the results we found in dorsal closure. We used the Mosaic Analysis with a Repressible Cell Marker (MARCM) genetic tool to express our Ed transgenes specifically in clones of *ed* mutant follicle cells (Lee et al., 2000).

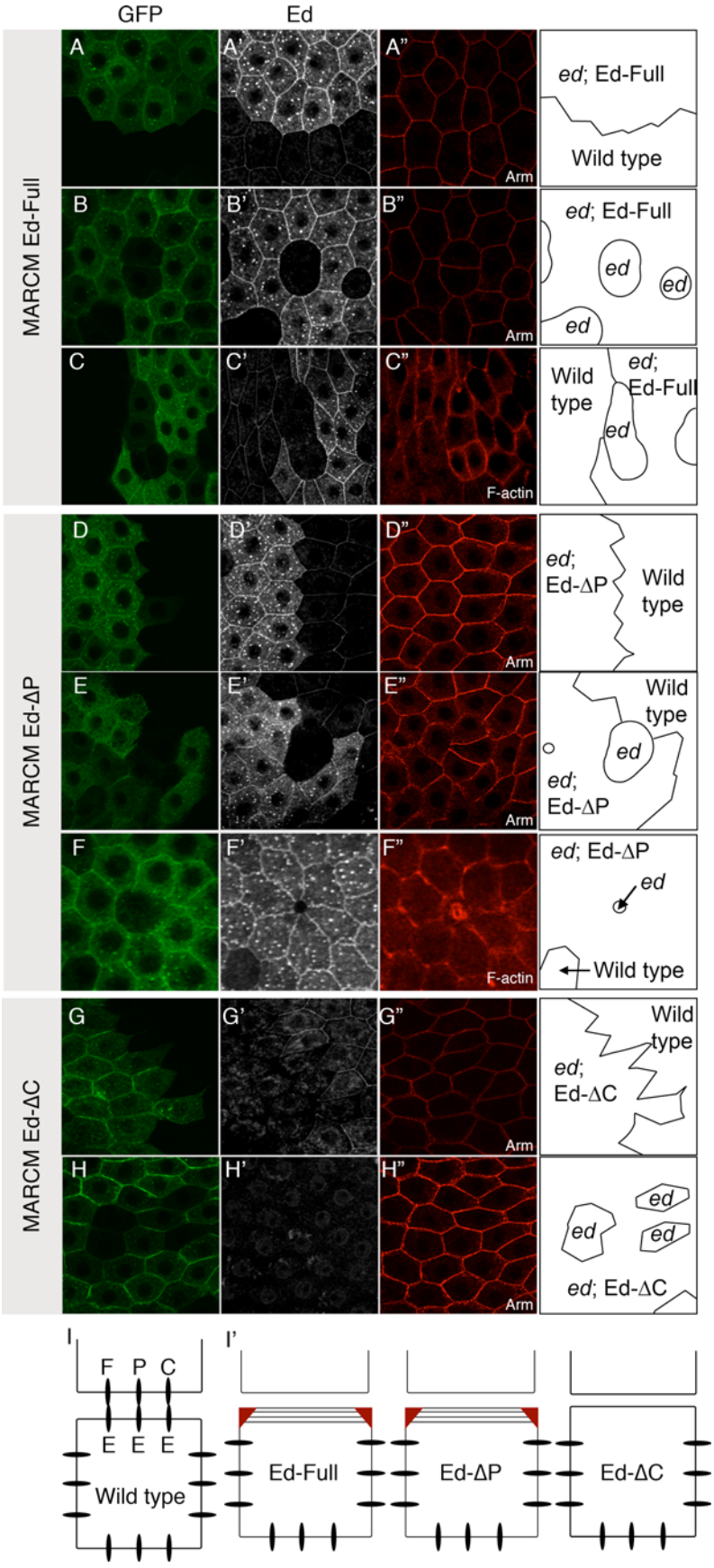
We first addressed whether the expression of our different *ed* transgenes in MARCM clones can stabilize Ed at the clone interface. When Ed-Full is expressed in *ed* mutant cells, Ed is detectable at the MARCM clone interface suggesting that

endogenous Ed and Ed-Full interact homophilically in *trans* at the clone border similarly to the Ed-LE embryos (Figure 3.6A-A'). Furthermore, the border of the Ed-Full MARCM clone with the non-mutant cells is jagged like normal cell-cell interfaces; the smooth border phenotype is rescued and no actomyosin cable is detectable at the clone border (Figure 3.6A"). These observations suggest that the maintenance of Ed at the clone border prevents the assembly of the actomyosin cable and rescues the smooth border phenotype.

When Ed-ΔP was expressed in MARCM clones, the contact between the Ed-ΔP MARCM cells with the wild type Ed expressing cells resulted in the presence of Ed at the interface further suggesting that homophilic interaction between endogenous Ed and Ed-ΔP *in trans* is sufficient to maintain Ed uniform around the cortex of the wild type cells contacting the MARCM clone (Figure 3.6D, D'). In addition, the MARCM clone interface with the wild type cell is jagged and does not assemble an actomyosin cable suggesting that Ed-ΔP can rescue the *ed* mutant clone border phenotype (Figure 3.6D").

Figure 3.6. The PDZ domain binding motif of Ed is dispensable for the formation of the actomyosin cable and stabilization of adherens junctions.

Diagrams to the right illustrate the different genotypes of the mosaic tissue. **A-C".** Follicular epithelia exhibiting Ed-Full expressing MARCM clones. **A, B, C.** Clones marked with GFP. **A', B', C'.** Ed. **A", B".** Arm. **C".** F-actin. **D-F".** Follicular epithelia exhibiting Ed-ΔP expressing MARCM clones. **D, E, F.** Clones marked with GFP. **D', E', F'.** Ed. **D", E".** Arm. **F".** F-actin. **G-H".** Follicular epithelia exhibiting Ed-ΔC expressing MARCM clones. **G, H.** Clones marked with GFP. **G', H'.** Ed. **G".** Arm. **H".** F-actin. **I.** Schematic representation of a wild type cell expressing endogenous Ed (E) contacting a cell expressing Ed-Full (F), Ed-ΔP (P) or Ed-ΔC (C). Endogenous Ed can interact homophilically with either of the three constructs. **I'.** Schematic representation of cells expressing Ed-Full, Ed-ΔP or Ed-ΔC (bottom) contacting an *ed* mutant cell (top). The juxtaposition of Ed-Full and *ed* mutant cells or Ed-ΔP and *ed* mutant cells triggers the formation of an actomyosin cable (triangles and lines). The juxtaposition of Ed-ΔC to *ed* mutant cells does not trigger the formation of an actomyosin cable.


GFP
Ed
MARCM Ed-Full
MARCM Ed-ΔP
MARCM Ed-ΔC
A
A'
A"
B
B'
B"
C
C'
C"
D
D'
D"
E
E'
E"
F
F'
F"
G
G'
G"
H
H'
H"
Arm
F-actin
ed; Ed-Full
Wild type
ed
ed; Ed-ΔP
ed; Ed-ΔC
I
I'
F P C
E E E
Wild type
Ed-Full
Ed-ΔP
Ed-ΔC

Finally, we expressed Ed-ΔC in MARCM clones and found that Ed-ΔC was sufficient to maintain endogenous Ed at the clone border, detectable with our antibody (Figure 3.6G, G'). As Ed-ΔC cannot be visualized with our Ed antibody, which was raised against the cytoplasmic tail, any immunostaining signal detected at the interface of the MARCM clone is therefore specific to the endogenous Ed protein on the wild type face of the clone border. Furthermore, the border of the Ed-Full MARCM clone with the non-mutant cell is jagged like normal cell-cell interfaces; the smooth border phenotype is rescued and no actomyosin cable is detectable at the clone border (Figure 3.6G"). These observations suggest that the extracellular domain of Ed is sufficient to maintain endogenous Ed by homophilic interaction in *trans*. Moreover, the loss of the phenotype indicates that maintaining endogenous Ed at the clone border prevents the assembly of the actomyosin cable and rescues the smooth border phenotype regardless of the presence of a cytoplasmic tail in the neighbouring cell.

Together these results confirm our previous observations found in dorsal closure; providing homophilic interaction in *trans* is sufficient to maintain endogenous Ed uniformly distributed around the cortex. Similarly to our results in dorsal closure, we observe that the asymmetric distribution of Ed around the cell cortex triggers the formation of an actomyosin cable. When Ed is maintained symmetrically distributed around the cell, no actomyosin cable assembles (Figure 3.6I).

Finally, the level of expression of Ed-Full is considerably higher than the endogenous levels expressed in the wild type cells, probably due to the use of the

tubulin promoter for the expression of the Ed transgene in the MARCM clones. Yet, the juxtaposition of wild type cells expressing the endogenous level of Ed to the MARCM clone cells overexpressing Ed does not result in the formation of actomyosin cable (Figure 3.6A'). This indicates that differences in the levels of Ed between neighbouring cells do not trigger the formation of the actomyosin cable. Rather, it is the juxtaposition of Ed expressing to Ed non-expressing cells that trigger the formation of the actomyosin cable.

3.4.8 The PDZ domain binding motif is dispensable for the formation of the actomyosin cable and for the stabilization of the adherens junctions

Currently, it is thought that Ed triggers the assembly of an actomyosin cable via the interaction of its PDZ domain binding motif with PDZ domain containing protein partners Baz and Cno. This model was built on a series of results obtained through a combination of *in vitro* binding analyses and co-immunoprecipitation experiments (Wei et al., 2005). These interactions prompted Wei and colleagues to propose a mechanism by which Ed stabilizes adherens junctions redundantly with the DE-cadherin/Armadillo complex and that the juxtaposition of *ed* mutant to Ed expressing cells triggered an actomyosin cable based on local destabilization of the adherens junctions. When MARCM clones are generated in the follicular epithelium, some *ed* mutant cells fail to express the transgene. We took advantage of these situations to generate interfaces between *ed* non-expressing cells and clones of *ed* mutant cells expressing our different Ed transgenes. Using this genetic tool we investigated the function of the PDZ domain binding motif and of the cytoplasmic tail *in vivo*.

When MARCM clones expressing Ed-Full contact Ed non-expressing cells, the transgene fail to be stabilized at the clone membrane and resulted in the formation of a smooth border and an actomyosin cable (Figure 3.6B-C"). This observation confirms that the differential expression of the Ed-Full transgene triggers the formation of an actomyosin cable like endogenous Ed and consequently that the Ed-Full transgene is functional (Figure 3.6I').

To determine the function of the PDZ domain binding motif, we analyzed situations where Ed-ΔP expressing *ed* mutant cells contacted *ed* mutant cells that failed to express the construct. This situation generated the differential expression of Ed-ΔP with Ed non-expressing cells. Surprisingly, when Ed-ΔP cells contacted Ed non-expressing cells, their interface was smooth and exhibited an actomyosin cable. This result indicates that the differential expression of Ed-ΔP is sufficient to trigger an actomyosin cable and therefore that the PDZ domain binding motif is dispensable for this function of Ed *in vivo* (Figure 3.6F-F"). Moreover, such clone borders exhibited the same phenotype of punctate adherens junctions observed when *ed* mutant cells contact wild type Ed expressing cells (Figure 3.6E-E"). These results were unexpected given that it had been previously proposed that the PDZ domain binding motif played a central role in the function of Ed for the stabilization of the adherens junctions and to prevent the formation of actomyosin cables (Lecuit, 2005; Wei et al., 2005). In contrast, our results indicate that the PDZ domain binding motif is dispensable for the formation of the actomyosin cable (Figure 3.6I'). Furthermore, although Ed might still interact with

Baz and Cno in certain situations, our results indicate that this interaction is not involved in the stabilization of adherens junctions.

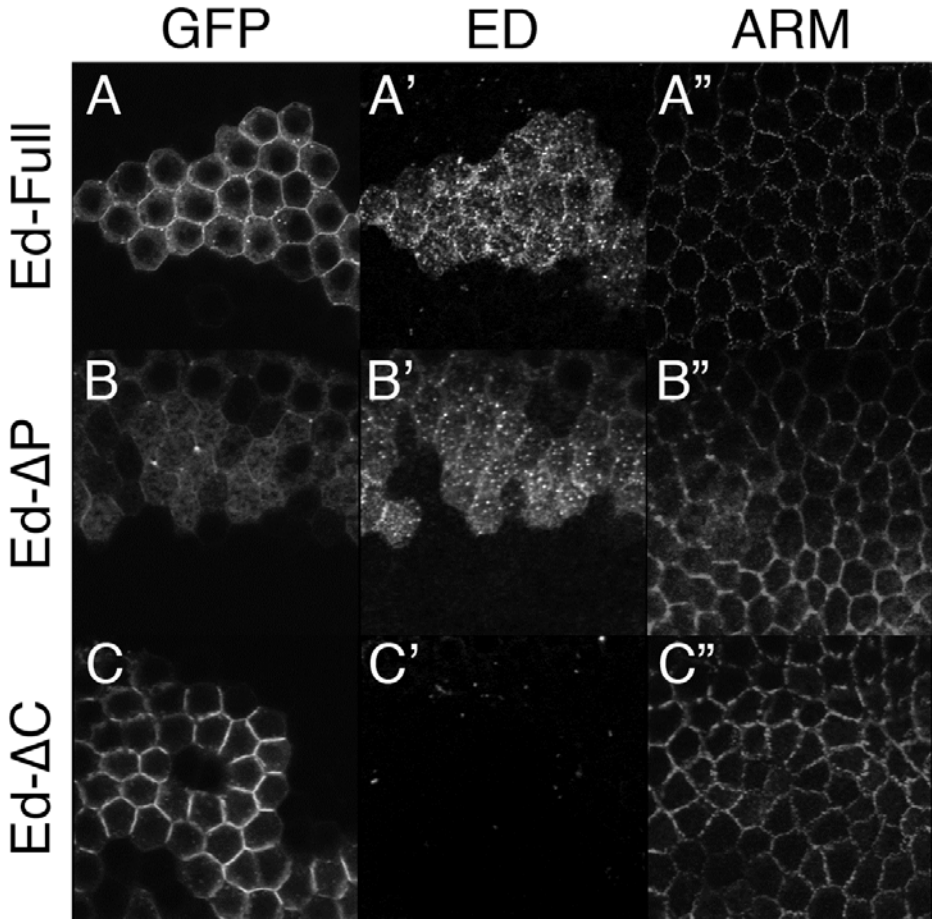
Finally, when *ed* mutant cells expressing Ed-ΔC are juxtaposed to *ed* mutant cells that do not express the transgene the clone border remains jagged and there is no actomyosin cable (Figure 3.6H-H"). Therefore, the differential expression of Ed-ΔC does not trigger the formation of an actomyosin cable (Figure 3.6I'). This indicates that the cytoplasmic tail of Ed is essential for the formation of an actomyosin cable and therefore that it contains at least one more functional domain besides the PDZ domain binding motif.

As an alternative approach to generate borders between Ed non-expressing cells and cells expressing our different transgene, we analyzed MARCM clones in stage 10A egg chambers. At that stage, Ed is not detectable in follicle cells and therefore clones of cells expressing Ed transgene at that stage contact wild type cells that lack Ed (Laplante and Nilson, 2006). When clones expressing Ed-Full during stage 10A were analyzed however, we observed that the transgenic protein remains in the cytoplasm and is not detected at the membrane (Figure S3.2A-A"). The same was observed with clones that expressed Ed-ΔP (Figure S3.2B-B"). This interesting outcome suggests the presence of a mechanism to prevent Ed-Full and Ed-ΔP from being embedded into the membrane during mid-oogenesis. This mechanism could also be responsible, at least in part, for the clearance of endogenous Ed during those stages.

In contrast, clones expressing Ed-ΔC during stage 10A exhibited Ed-ΔC at the membrane. The border of those clones did not exhibit an actomyosin cable and were jagged (Figure S3.2C-C"). This observation further suggests that the cytoplasmic tail of Ed is essential to trigger the formation of an actomyosin cable and to make the clone border smooth.

Supplemental Figure 3.2. Ed-Full and Ed-ΔP but not Ed-ΔC localize in the cytoplasm during stage 10A.

Stage 10A egg chambers with clones expressing Ed transgenes. **A-A".** MARCM clone expressing Ed-Full marked with GFP (A) exhibit cytoplasmic Ed localization (A'). The outlines of the cells are marked with Arm (A"). **B-B".** MARCM clone expressing Ed-ΔP marked with GFP (B) exhibit cytoplasmic Ed localization (B'). The outlines of the cells are marked with Arm (B"). **C-C".** MARCM clone expressing Ed-ΔC-GFP, which is associated with the membrane (C). At that stage, endogenous Ed is not detectable by immunostaining (C'). The outlines of the cells are marked with Arm (C").


GFP
ED
ARM
Ed-Full
Ed-ΔP
Ed-ΔC
A
A'
A"
B
B'
B"
C
C'
C"

3.5 Discussion

3.5.1 The asymmetric distribution of Ed establishes the planar polarity of the actin cytoskeleton

The localized regulation of the actin cytoskeleton within cells is crucial for exerting directed tension and provides the necessary forces for cytokinesis, single and multicellular cell migration. How do neighboring cells coordinate their cytoskeleton during epithelial sheet movements? During *Drosophila* dorsal closure, the leading cells of the migrating epidermis assemble a local actomyosin cable at their front. In this work we have shown that the asymmetric distribution of Ed establishes the planar polarity of the cells to localize regulators of the actin cytoskeleton to the migrating front during the concerted movement of epidermal cells in dorsal closure.

Prior to dorsal closure, Ed expression is lost from the amnioserosa cells. In the absence of a homophilic binding partner, Ed disappears from the leading edge of the dorsal-most epidermal cells where they contact the Ed non-expressing amnioserosa creating an asymmetric distribution of Ed along the dorsal-ventral axis. When Ed is asymmetrically distributed, the actomyosin cable assembles at the leading edge visible by the accumulation of F-actin and myosin II (pMLC and MHC). Moreover, the actin regulators RhoGEF2, Dia and Ena also accumulate locally along the leading edge. To study the function of the distribution of Ed, we compared the localization of actin regulating molecules in the dorsal-most epidermal cells between dorsal-most epidermal cells that expressed either wild type asymmetric distribution of Ed, no Ed at all ($ed^{M/Z}$),

and uniform Ed distributed symmetrically around the cortex (Ed-LE). Our results show that the loss of differential Ed expression prevents the accumulation of RhoGEF2, Dia and Ena as well as the assembly of the actomyosin cable. From our work, we propose that the distribution of Ed provides a planar polarity cue that results in the local activation of the machinery that generates the actomyosin cable.

Our results also demonstrate that the proper establishment of the ANCs requires the asymmetric distribution of Ed in the dorsal-most cells. Indeed the accumulation of Ena and Dia occurs at the ANC in wild type cells when Ed is asymmetrically distributed but not when this distribution is abrogated by the removal of Ed or by restoring the localization of Ed to the leading edge. We propose that the asymmetric distribution of Ed triggers the assembly of functional ANC.

The Rho1 activator RhoGEF2 accumulates at the leading edge suggesting that Rho1 is locally activated at the leading edge during dorsal closure. Consistent with this observation, Dia, an effector of Rho1 becomes enriched at the ANCs where F-actin is initially polymerized. Therefore, we posit that the planar polarity established by the asymmetric distribution of Ed within the dorsal-most epidermal cells triggers the assembly of an actomyosin cable at the leading edge by the local activation of the Rho1 pathway.

The Wg signaling pathway is involved in the establishment of the planar polarity in the dorsal-most epidermal cells. This planar polarity signal influences the

localization of the actin cytoskeleton and is responsible for the formation of the ANC (Kaltschmidt et al., 2002). It is possible the Wg pathway might regulate the expression profile of Ed thus affecting its distribution around the dorsal-most epidermal cells. However, Wg influences the orientation of the microtubule network and the distribution of the planar polarity core complex and septate junctions around the dorsal-most epidermal cells (Kaltschmidt et al., 2002). In contrast, the distribution of Ed does not affect the organization of those other planar polarity markers and appears specific to the actin cytoskeleton. Therefore, the Wg pathway likely acts upstream of Ed in the establishment of the planar polarity during dorsal closure.

3.5.2 The role of the actomyosin cable during dorsal closure

Ed-LE embryos represent a genetic situation in which the formation of the actomyosin cable is abrogated and an ideal model for the study of the role of the actomyosin cable during dorsal closure. Other works have investigated the role of the actin cable by studying embryos mutant for genes involved in actin regulation, which have more diverse and deleterious effects than Ed-LE (for example (Franke et al., 2005; Magie et al., 1999)). Ed-LE embryos close almost completely at the dorsal midline leaving only a small gap in the epidermis. Therefore the tension provided by the actomyosin cable is dispensable for the closure of the epidermis probably due to the combined effort of the other forces that contribute to dorsal closure, namely the constant constriction of the amnioserosa and the elongation of the lateral epidermis,.

What other function could the actomyosin cable fulfill? The observation of the leading edge in wild type embryos and Ed-LE embryos during dorsal closure suggests that the tension in the actomyosin cable maintains the leading edge taut and restricts the contact of the two sheets of epidermis to the anterior and posterior canthi. At those sites, the zippering of the epidermis aligns the two sheets with a cell-by-cell accuracy, a process based on a yet unknown mechanism that confers a distinct identity to the epidermal cells and prevents cells within a segment from joining with the wrong partner (Millard and Martin, 2008). However, the same cell identification mechanism is used from segment to segment and therefore, if the mismatch is great enough, cells between segments can contact the wrong target. Therefore, the tension provided to the leading edge by the contractile activity of the actomyosin cable could function to prevent large disparity in the alignment of the sheets by maintaining the only two zippering fronts one at the anterior and one at the posterior thus bringing few cells into contact at once and minimizing the possibility of mismatch errors.

3.5.3 The PDZ domain binding motif is dispensable for the formation of the actomyosin cable

The current model of Ed function emphasizes the interaction between the PDZ domain binding motif of Ed and the PDZ domain of the actin interacting protein Cno and the polarity protein Baz (Wei et al., 2005). This model is based on *in vitro* binding assays and co-immunoprecipitation experiments. Our work however indicates that the PDZ domain binding motif is dispensable both for triggering the formation of the actomyosin cable and for stabilizing adherens junctions *in vivo*. While our results

dismiss the role for the PDZ domain binding motif of Ed in this context they do not exclude the possible interaction between Ed and Baz or Cno in other contexts. Alternatively, as Ed is enriched at adherens junctions, it is possible that the co-immunoprecipitation of Ed from whole tissues isolated other proteins enriched at adherens junctions and since many of them contain PDZ domains this technique can lead to false positive interactions. *In vivo* analyses will be essential to verify the multiple interactions suggested by molecular experiments. Indeed, such experiments have so far stretched the list of PDZ domain containing interactors to include Cno, Baz, *Drosophila* Glutamate Receptor Interacting Protein (DGrip) and Jaguar/Myosin VI (Lin et al., 2007; Swan et al., 2006; Wei et al., 2005). Although. it is possible that Ed is very promiscuous in its interaction, such interactions should be justified by *in vivo* analyses.

3.5.4 The expression of Ed is downregulated during the development of different epithelia

During the course of our work, we found that the expression or the localization of Ed transgenes was prevented in certain cells during specific stages of development. In the embryo, the expression of Ed-Full and Ed-ΔP was prevented in the first few rows of amnioserosa cells, named the peripheral amnioserosa cells. In the egg chamber during stages 10A-10B, Ed-Full and Ed-ΔP are not embedded in the membrane. These observations suggest the presence of mechanisms that prevent Ed from being present at the membrane of those cells during specific developmental stages and also recognize transgenic Ed proteins. Interestingly, Ed-ΔC, which itself cannot trigger the formation of actomyosin cables, passes such mechanisms unnoticed.

What mechanisms could we predict regulate the expression of Ed? During embryogenesis, the amnioserosa cells closest to the epidermis adopt a specialized fate, the peripheral amnioserosa cell fate. These cells are different from the other amnioserosa cells within the tissue and are closest to signals such as the Dpp morphogen secreted from the dorsal-most rows of epidermal cells. These cells also express a specific enhancer trap, pAS-Gal4, the expression of which is dependent on receiving Dpp (Wada et al., 2007). Therefore, it is possible that a mechanism instructed in their special fate is responsible for recognizing Ed and clearing it on time prior to dorsal closure for the actomyosin cable to assemble.

The signal pathways and molecular mechanism that regulate the broad range of cellular events occuring to the follicular epithelium during the mid-stage of oogenesis are deceptively un-characterized. However, it can be assumed that a particular signal or the combination of different signals is responsible for the dramatic rearrangement of the follicular epithelium during that stage. Such signals could be responsible for the interruption of the expression of Ed and ensure the absence of Ed during those stages.

3.5.5 Reciprocal distribution of Ed/Baz and actin cytoskeleton

The polarity Baz exists in a complex with Par-6 and atypical protein kinase C (aPKC) and has long been studied for its effect on the polarization of the C. elegans embryo and the apical-basal polarity in epithelial cells (Lin et al., 2000; Munro, 2006;

Suzuki and Ohno, 2006). In *Drosophila* embryogenesis during germ band extension, Baz and MHC are enriched at reciprocal faces of ectodermal cells (Zallen and Wieschaus, 2004). MHC accumulates at the anterior and posterior faces of the cell where constriction occurs while Baz accumulates at the dorsal and ventral faces of the cell where new cell contacts are formed. As Baz regulates the assembly of adherens junctions, it was suggested that it recruits new adherens junctions at the expanding face of the cell. Yet, the link between Baz and the actin cytoskeleton is still nebulous. However, in the cellularizing embryo, Baz stabilizes the nascent adherens junctions via its interaction with Bitesize, a synaptotagmin-like protein and Moesin, which reorganizes the actin cytoskeleton into a circumferential actin belt (Pilot et al., 2006).

The complementary localization of Baz and MHC is recapitulated in the dorsal-most epidermal cells during dorsal closure. Our work shows that the polarized distribution of both the actin cytoskeleton and Baz is dependent on the asymmetric distribution of Ed. The recurrence of this reciprocal distribution within cells that undergo a shape change that require localized subcellular tension raises a simple question: what it the relation between the distribution of Ed and that of Baz and myosin II? One possibility is that the distribution of Ed regulates the distribution of both Baz and MHC independently. However, another possibility is that Ed regulates the distribution of Baz, which in turns influences the actin cytoskeleton. In support of this hypothesis is the recent finding that the Par-3/Par-6/aPKC complex inhibits the activation of RhoA via p190RhoGAP in mammalian dendritic cells (Zhang and Macara, 2008). Therefore, in dorsal closure, the loss of Baz from the leading edge could allow

the activation of Rho1 and thus the assembly of the actomyosin cable. Alternatively, the distribution of Ed could regulate regulators of the actin cytoskeleton, which in turn regulate the localization of Baz. This hypothesis is also supported by recent work on mammalian culture cells. In this work, it was found that RhoA phosphorylates Par-3 causing the disassembly of the Par-3/Par-6/aPKC complex at the leading edge (Nakayama et al., 2008). If this were true during dorsal closure, the activation of Rho1 at the leading edge could explain the destabilization and loss of Baz from the leading edge. In either cases, the polarity Par complex influences or is influenced by the Rho small GTPase and further work is required to determine the link between the *Drosophila* Baz/Par-6/aPKC complex and the actin cytoskeleton.

3.6 Conclusion

Our work identifies a novel mechanism for the planar polarization of the actin cytoskeleton and polarity protein Baz in the dorsal-most epidermal cells during dorsal closure based on the asymmetric distribution of Ed. In the embryo, the asymmetric distribution of Ed results in the accumulation of RhoGEF2, Dia and Ena at the leading edge and the subsequent formation of the actomyosin cable. The abrogation of this polarized distribution either by maintaining Ed uniformly distributed or by removing it completely in the dorsal-most epidermal cells prevents the polarized accumulation of the actin regulators and the formation of the actomyosoin cable. Furthermore, our work indicates that the PDZ domain binding motif of Ed is dispensable for its function in the assembly of actomyosin cables. This mechanism initiated by the differential expression

of a cell adhesion molecule may represent a general mechanism for the localized regulation of the actin cytoskeleton during epithelial morphogenesis.

3.7 Acknowledgements

We thank Richard Fehon, David Hipfner, Daniel Kiehart, Steve Rogers, Steve Wasserman, Daitsuke Yamamoto and Jennifer Zallen for fly strains and antibodies. Monoclonal antibodies were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA. All microscopy was performed at the CIAN at McGill University. This work was supported by the Natural Sciences and Engineering Research Council of Canada. C.L. was supported by the National Cancer Institute of Canada (NCIC).

Chapter 4: Discussion

4.1 Ed polarizes the actin cytoskeleton

The polarization of actin dynamics during tissue morphogenesis ensures directionality to the migration front. But how do migrating cells acquire a planar polarized actin cytoskeleton? In the course of my research, I characterized the role of the homophilic-binding cell adhesion molecule Ed in the formation of actomyosin cables in *Drosophila* epithelia. The cortical distribution of Ed is regulated by its homophilic-binding property and by extension is dependent on the expression of Ed in the neighbouring cells; Ed expressing cells exhibit a lack of Ed at the face that contacts a non-expressing neighbour thus resulting in the asymmetric distribution around its cortex. My work shows that the asymmetric distribution of Ed around the cell cortex acts as a planar polarity cue. This signal in turns influences the cortical distribution of actin regulators and triggers the formation of an actomyosin cable at the leading edge of tissue movement (Chapter 3).

4.1.1 Polarized Ed distribution triggers the formation of actomyosin cables

During embryonic dorsal closure, an epidermal gap, covered by the amnioserosa, is closed by the dorsal migration and suturing of the two sheets of epidermis at the dorsal midline. The epidermis expresses Ed and contacts the Ed non-expressing amnioserosa. At this border, the dorsal-most cells of the epidermis lack Ed at the face that contacts the amnioserosa, which is referred to as the leading edge of migration.

This asymmetric distribution of Ed polarizes the dorsal-most epidermal cells across the plane of the tissue and this planar polarity is marked by the local re-distribution of the actin cytoskeleton in those cells. Indeed, the asymmetric distribution of Ed results in the localized accumulation of actin regulators, RhoGEF2 and Dia along the leading edge, as well as the formation of the actomyosin cable marked by the enrichment of F-actin, phosphorylated MLC and MHC also at the leading edge (sections 3.4.3 and 3.4.4).

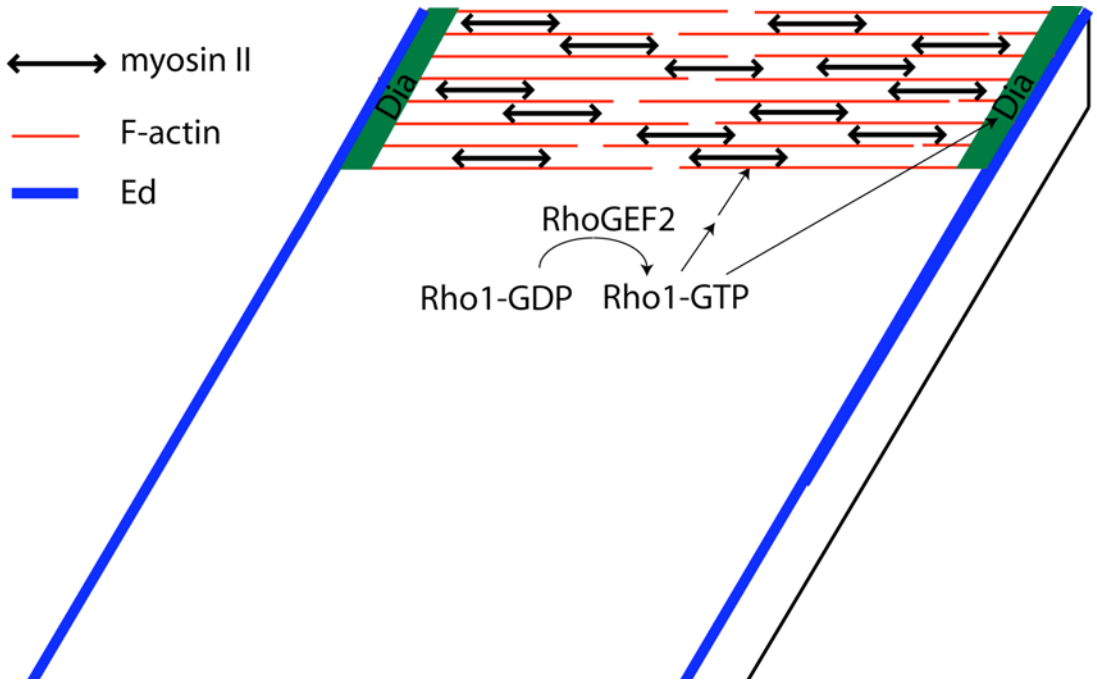
How does the distribution of Ed around the dorsal-most epidermal cells trigger the formation of an actomyosin cable? The local accumulation of the Rho1 activator RhoGEF2 at the leading edge suggests that the asymmetric distribution of Ed regulates the local activation of the Rho small GTPase Rho1 at the leading edge. Based on the known functions of Rho1, it can be predicted that the local activation of Rho1 initiates the formation of the actomyosin cable. Consistent with this hypothesis, Rho1 activity is essential for the formation of the actomyosin cable as embryos expressing Rho-DN or mutant for *rho1* display a defective actomyosin cable (Bloor and Kiehart, 2002; Magie et al., 1999). Moreover, Rho1-GTP binds to and activates Diaphanous-type formins by the relief of an intramolecular inhibitory interaction between the amino- and carboxy-termini of Dia. The release of Dia from this self-inhibition allows it to homodimerize and nucleate actin filaments (Pollard, 2007). During dorsal closure, F-actin is initially enriched along the leading edge at tricellular junctions where two epidermal cells contact the amnioserosa (Kaltschmidt et al., 2002). These sites, named actin-nucleating centers (ANCs), are also enriched with Dia. Therefore, a sensible prediction is that the enriched Dia at ANCs is activated by Rho1 and can thus initiate the polymerization of

unbranched F-actin. F-actin is then elongated and crosslinked into bundles that run along the face of the leading edge, producing the actin backbone of the actomyosin cable. Furthermore, the activation of Rho small GTPases results in the activation of myosin II. Rho1-GTP can activate myosin II by regulating the phosphorylation state of the myosin II regulatory light chain, MLC. The activation of Rho1 at the leading edge can activate the phosphorylation of MLC by Rok or MLCK and prevent its de-phosphorylation by inactivating myosin II phosphatase (Adelstein et al., 1978; Amano et al., 1996; Kimura et al., 1996). The resulting active myosin II produces the tension in the actomyosin cable by sliding antiparallel filaments of actin in opposite directions (Figure 4.1).

Therefore, the differential expression of Ed between the amnioserosa and the epidermis results in the asymmetric distribution of Ed in the dorsal-most epidermal cells. This in turn establishes an aspect of the planar polarity of those cells. This planar polarity is essential for the accumulation of actin regulators to the leading edge and triggers the assembly of the actomyosin cable at the leading edge, most likely via the local activation of Rho1 GTPase. However, the distribution of Ed does not dictate the entire planar polarity of the dorsal-most epidermal cells as the septate junctions, the component of the planar polarity core complex Flamingo and the microtubule network all adopt a planar polarized distribution independently of Ed.

Figure 4.1. The asymmetric distribution of Ed triggers the assembly of an actomyosin cable via the localized activation of Rho1 at the leading edge.

Schematic representation of a dorsal-most epidermal cell during dorsal closure. The asymmetric distribution of Ed in the cell (blue) triggers the accumulation of RhoGEF2 at the leading edge, which activates Rho1. Active Rho1-GTP activates the formin Dia at ANCs (green), which nucleates the formation of F-actin (red). Rho1-GTP activates myosin II (black).


myosin II
F-actin
Ed
Dia
Dia
RhoGEF2
Rho1-GDP
Rho1-GTP

4.2 Ed distribution triggers the formation of the actomyosin cable during appendage tube floor closure

During oogenesis, groups of follicle cells rearrange from a flat epithelium into appendage secreting tubes known as the dorsal appendages. The molecular changes that occur during these morphogenetic movements have not been investigated thoroughly (Dorman et al., 2004). Yet, we know that the two types of cells that participate to this morphogenesis, the floor and roof cells express Ed differentially; Ed is expressed in the floor cells, which flank the Ed non-expressing roof cells (section 2.4.6). An actomyosin cable assembles at this interface and in the absence of this actomyosin cable the floor of the tube fails to close (see section 2.4.7). Therefore, we can predict that the function of Ed in the assembly of the actomyosin cable at the interface of the roof and floor of the tube to be similar to its role during dorsal closure. It is likely that the asymmetric distribution of Ed in the floor cells polarizes their actin cytoskeleton and results in the accumulation of RhoGEF2 and Dia at the leading edge of the floor cells where they contact the roof cells. Further investigation will reveal whether this mechanism is indeed involved in the closure of the appendage tube floor.

4.3 Ed is a potential negative regulator of non-muscle Myosin II

Differential expression of Ed between juxtaposed groups of cells results in the asymmetric distribution of Ed within the Ed expressing cell. This polarized distribution of Ed controls the aspect of the planar polarity of the cells that concerns the actin

cytoskeleton thereby triggering the assembly of an actomyosin cable at the Ed-free edge. How could the distribution of Ed control the actin cytoskeleton? One possibility is that Ed could act as a negative regulator of Rho1 thus preventing the formation of an actomyosin cable where Ed is present around the cell. Consistent with this hypothesis are the isotropic constriction of the presumptive roof of the dorsal appendage primordia in the egg chamber and that of the amnioserosa cells in the embryo. In both cell types, the cells lack Ed and constrict apically. Moreover, pMLC is sometimes enriched around the cortex of *ed* mutant follicle cells (CL unpublished results) and amnioserosa cells that ectopically express Ed-full exhibit a reduced cortical localization of MHC (CL unpublished results). Ectopic expression of Ed-ΔC in the amnioserosa does not affect the cortical distribution of MHC. Finally, MARCM clones mutant for *ed* but expressing Ed-ΔC still display enriched cortical pMLC. Yet, ectopic expression of Ed-Full in the amnioserosa does not prevent those cells from constricting suggesting that although Ed-full might prevent the cortical enrichment of MHC, another mechanism enables the cells to constrict.

The orientation of the microtubule network in wounded Xenopus oocyte is crucial for the formation of a contractile actomyosin cable at the edge of the wound (Mandato et al. 1999; Mandato and Bement, 2003). In that system, microtubules, oriented perpendicular to the edge of the wound, bring short actin filaments to the wound edge where they are presumably incorporated into the constricting actomyosin ring. During dorsal closure in the dorsal-most epidermal cells, the microtubules are oriented perpendicular to the leading edge and to the actomyosin cable (Jankovics and Brunner,

2006). Therefore, one other possible function for Ed would be to provide a planar polarity cue that would orient the microtubules thus helping the formation of the actomyosin cable. This hypothesis is however unlikely since the microtubule network is properly oriented when Ed is absent or when it is distributed symmetrically in the dorsal-most epidermal cells. Moreover, it was shown that the dorsal closure actomyosin cable assembles in the absence of microtubules (Jankovics and Brunner, 2006).

4.4 The role of the actomyosin cable

During morphogenetic events in oogenesis and embryogenesis, the differential expression of Ed between juxtaposed tissues generates an actomyosin cable at their interface, which corresponds to the leading edge of migration. During embryonic dorsal closure, the differential expression of Ed triggers the assembly of an actomyosin cable at the leading edge of the dorsal-most epidermal cells. What is the function of the actomyosin cable during dorsal closure? The cable is thought to provide one of the forces that draw the epidermal sheets close at the dorsal midline, analogous to the action of a purse string. Cutting the actomyosin cable by laser microsurgery revealed that the actomyosin cable is indeed under tension and thus may provide one of the forces that contribute to the closure of the epidermis (Kiehart et al., 2000). What happens to dorsal closure when the cable is completely absent? Embryos mutant for genes that encode different components of the actin cytoskeleton such as *zipper* (coding for MHC) and *rho1* exhibit defective assembly of the actomyosin cable. Yet, the sheets of epidermis are still drawn dorsally and join albeit imperfectly leaving gaps along the dorsal midline (Magie et al., 1999; Young et al., 1993). These results indicate that other forces

compensate for the role of the actomyosin cable in closing the epidermal sheets, namely the constant constriction of the amnioserosa and the elongation of the epidermal cells along the dorsal-ventral axis. Similarly, embryos that maintain Ed uniformly distributed around the dorsal-most epidermal cells via the ectopic expression of an Ed transgene in the amnioserosa (Ed-LE embryos) fail to assemble an actomyosin cable (section 3.4.3). Using this system we can determine the role of the actomyosin cable during dorsal closure without affecting other aspects of the cells' functions unlike using mutations in *rho1* or *zipper*. Ed-LE embryos close almost completely at the dorsal midline leaving only a small gap in the epidermis (section 3.4.3). Therefore the tension provided by the actomyosin cable is dispensable for the closure of the epidermis presumably due to the combined effort from the constant constriction of the amnioserosa and the elongation of the lateral epidermis.

What other function could the actomyosin cable fulfill? Observations of the leading edge in wild type and Ed-LE embryos during dorsal closure suggest that the tension in the actomyosin cable maintains the leading edge taut and restricts the contact of the two sheets of epidermis to the anterior and posterior canthi (section 3.4.1). At those sites, the zippering of the epidermis aligns the two sheets with a cell-by-cell accuracy (Jacinto et al, 2002). This process is based on a yet unknown mechanism that confers a distinct identity to the epidermal cells and prevents cells within a segment from joining with the wrong partner (Millard and Martin, 2008). The work of Millard and Martin proposes that the same cell identification mechanism is re-used from segment to segment and therefore, if the mismatch is great enough, cells from

neighbouring segments can contact the wrong target. Given these results, my work proposes that the tension provided to the leading edge by the contractile activity of the actomyosin cable could function to prevent large disparity in the alignment of the sheets by maintaining the only two zippering fronts one at the anterior and one at the posterior thus bringing few cells into contact at once and minimizing the possibility of mismatch errors. To test this hypothesis, Ed-LE embryos could be analyzed at the end of embryogenesis, once dorsal closure is normally complete, to determine whether such embryos exhibit a higher frequency of mismatched segments than wild type embryos. A greater frequency of misaligned segments is to be expected in Ed-LE embryos compared to wild type embryos if the actomyosin cable prevents the mismatching of segments.

The molecular events that occur during the formation of the appendage secreting tubes of the egg chamber are still uncharacterized. However, the similarities between appendage tube closure and embryonic dorsal closure suggest that the formation of tubes from the flat follicular epithelium also requires the combination of multiple forces. Indeed, an actomyosin cable assembles between the floor and roof cells, the two cell types that will populate the tube. In addition, the roof cells constrict their apices similarly to the apical constriction of the amnioserosa. Finally, the floor cells elongate under the roof cells reminiscent of the elongating epidermis along the dorsal-ventral axis. The assembly of the actomyosin cable requires the differential expression of Ed between the two cell types and is essential for the closure of the floor of the tube. In the absence of the actomyosin cable, the floor cells fail to close leaving a gap between the anterior and medial portions of the floor suggesting that the actomyosin cable draws the

two parts of the tube floor together and allow them to zip shut at the midline (Laplante and Nilson, 2006).

The recurrent use of the differential expression of Ed in the production of actomyosin cables during morphogenetic events suggests that it may represent a general mechanism utilized to trigger the assembly of an actomyosin cable in a variety of other circumstances. Actomyosin cables are used in adult epithelia namely for the closure of wounds and the extrusion of apoptotic cells (Bement, 2002). As the asymmetric distribution of Ed is central to the formation of the actomyosin cable during dorsal closure, a process often compared to wound healing; it seems likely that Ed would participate in the formation of the actomyosin cable during wound healing. The distribution of Ed in the cells neighbouring the edge of a wound could be asymmetric with Ed lacking from the wound edge and present at the other faces of the cells. Previous studies have shown that *Drosophila* embryos wounded by laser treatment heal via the formation of both a contractile actomyosin cable and filopodia extension at the wound edge (Wood et al., 2002). To determine whether the distribution of Ed is essential to the process of wound closure, embryos that completely lack Ed, $ed^{M/Z}$ embryos, expressing a GFP-moesin construct could be wounded by laser treatment and the closure of the wound followed live by confocal microscopy. GFP-moesin, which highlights filamentous actin is a common tool used to visualize the actomyosin cable in live imaging of dorsal closure and is therefore expected to highlight the actomyosin cable at the wound edge (Edwards et al., 1997). The dynamics of closure could then be compared to closure of wounds in wild type embryos expressing GFP-moesin.

Actomyosin cables also participate in the extrusion of apoptotic cells from epithelial tissues. Dying cells are extruded from healthy epithelia to prevent the formation of a gap in the tissue and thus maintaining the homeostatic properties of the epithelium. The extrusion of apoptotic cells occurs at least in part via the contractile action of an actomyosin cable in the cells neighbouring the dying cell (Rosenblatt et al., 2001). The follicular epithelium is a powerful model tissue to study the process of apoptotic cell extrusion. Starting late in oogenesis until the egg is laid, follicle cells undergo apoptosis and one-by-one are cleared from the epithelium. When a follicle cell dies, an actomyosin cable assembles in the neighbouring cells at the face that contact the dying cell (C.L. unpublished observation). Interestingly, components of adherens junctions such as Armadillo, DE-cadherin and Ed are downregulated at the face of the healthy cells that contact the apoptotic cell (C.L. unpublished observation). Is the differential expression of Ed required for the formation of that actomyosin cable? To answer this question, clones of *ed* mutant follicle cells in late stage egg chambers could be analyzed for the formation of an actomyosin cable at the interface of apoptotic cells. Apoptotic cells are easily labeled using commercially available markers, while the actomyosin cable could be marked with fluorescently labeled phalloidin to highlight F-actin. The asymmetric distribution of Ed detected at the edge of an apoptotic cell might trigger the assembly of the actomyosin cable at the edge contacting the dying cell in which case, *ed* mutant cells surrounding an apoptotic cell would not assemble an actomyosin cable at the face contacting the dying neighbour.

4.5 The fascinating expression profile of Ed

4.5.1 Downregulation of Ed in the amnioserosa during embryogenesis

The proper regulation of Ed expression prior to morphogenetic movements is crucial for generating the differential expression of Ed at the interfaces and is therefore responsible for the formation of the actomyosin cable. During embryogenesis, Ed is initially expressed in all the cells until stage 11 when the amnioserosa downregulates the expression of Ed. What could cause this change in the expression profile of Ed in the amnioserosa? The JNK and Wg signaling pathways are two candidate pathways that are known to influence cell shape changes and morphogenesis during dorsal closure and could therefore affect the expression profile of Ed.

Prior to dorsal closure, JNK signaling is turned off in the amnioserosa by the independent activity of Hindsight and Puckered, two negative regulators of JNK activity, but remains high in the dorsal-most epidermal cells (Reed et al., 2001). Therefore, the JNK pathway seems a likely candidate to regulate the expression of Ed during embryogenesis as reduction of JNK signaling in the amnioserosa could be responsible for the decrease in Ed expression. Yet, the expression of Ed in both the amnioserosa and epidermis is unaffected in different JNK pathway mutants (C.L. unpublished results). Moreover, the profile of JNK activity during dorsal closure is unaffected by the loss of Ed (Lin et al., 2007). Therefore, the distribution of Ed and the JNK signaling pathway represent two essential but independent signals for the process of dorsal closure.

The link between the Wg signaling pathway and the distribution of Ed has not yet been investigated. The Wg pathway regulates multiple aspects of the planar polarity in the dorsal-most epidermal cells during dorsal closure namely the organization of the actin cytoskeleton and the microtubule network as well as the distribution of septate junctions and the planar polarity core complex around the dorsal-most epidermal cells (Kaltschmidt et al., 2002). It is possible that Ed is one of the multiple downstream effectors of the Wg pathway. To test this hypothesis, the profile of Ed expression prior to and during dorsal closure could be investigated in *wg* mutant embryos. If Wg is responsible for establishing the differential expression of Ed between the amnioserosa and epidermis, it is predictable that Ed would be detected in the amnioserosa of *wg* mutant embryos or Ed would be lost from the epidermis during dorsal closure. In either scenario, Ed would be uniformly distributed around the dorsal-most epidermal cells thus resulting in defective planar polarity of the actin cytoskeleton.

4.5.2 Downregulation of Ed in the follicular epithelium during mid-oogenesis and in the roof cells during appendage floor closure

Starting during stage 8 of oogenesis, the levels of detectable Ed in the follicle cells decrease until Ed is undetectable throughout the epithelium by stage 10A (section 2.4.5). What signal could be responsible for the downregulation of Ed during mid-oogenesis? Unfortunately, the lack of information about the signals and effectors that control those morphogenetic events leaves us with few answers. In addition, why do the follicle cells downregulate the expression of Ed during those stages? Part of the answer may lie in the dramatic cellular rearrangements that characterize this period of oogenesis. During

stage 9, the follicular epithelium undergoes a series of cell shape changes that result in the alignment of the posterior ~600 cells on top of the oocyte while the remaining ~50 anterior follicle cells stretch to cover the nurse cells. One possible reason for the removal of Ed during that period is that the presence of Ed might prevent the changes in the cell shape inhibiting the morphogenetic process. Yet, ectopic Ed expressed during stage 8 and 9 localizes to the membrane and does not disrupt the posterior migration of the follicular epithelium (C.L. unpublished results). However, ectopic Ed expressed in the follicle cells during stage 10A is not detectable at the membrane but instead is visible as a cytoplasmic haze suggesting a post-translational regulation (section 3.4.8). An alternative reason for the downregulation of Ed during mid-oogenesis could be to clear Ed from the epidermis in order to establish the particular expression profile of Ed during stage 10B of oogenesis.

After the downregulation of Ed during mid-oogenesis, Ed becomes detectable in a subset of follicle cells organized in a T-shape at the dorsal-anterior region during stage 10B. By stage 11, Ed has reappeared in all the follicle cells except for two populations of cells located on either side of the dorsal midline, the roof cells (section 2.4.6). What could prevent the expression of Ed in the roof cells? The roof cells express high levels of the transcription factor Broad while Broad is not expressed in the cells of the midline (Deng and Bownes, 1997; Tzolovsky et al., 1999). This expression of Broad is complementary to that of Ed and therefore suggests that Broad could control the expression of Ed in the roof cells either directly or indirectly via instructive signals needed for roof cell fate. To test this hypothesis, the levels of Ed expression could be investigated in clones of *broad* mutant cells affecting the roof cell population. If Ed is

expressed in *broad* mutant roof cells, this indicates that Broad regulates directly or indirectly the expression of Ed. Alternatively, Ed expression could be analyzed in clones of cells expressing ectopic high Broad. If Ed levels are increased in such clones, it would again suggest that Broad influences the expression of Ed directly or indirectly via downstream regulators.

4.6 Dissecting Ed: the function of the different domains of Ed

4.6.1 The extracellular domain of Ed regulates its cortical distribution

The extracellular domain of Ed is essential for the homophilic interaction and therefore the distribution of Ed around the cell cortex (sections 3.4.1, 3.4.7). The extracellular domain of Ed can therefore be considered as a regulatory domain that positions the cytoplasmic tail of Ed around the cell cortex and thus influences its activity. To determine the function of the extracellular domain of Ed, a transgene coding for an extracellular domain truncation variant of Ed or a chimeric protein fusion between the cytoplasmic tail of Ed and the extracellular domain of an unrelated transmembrane protein could be generated and tested for their function in the formation of the actomyosin cable.

The extracellular domain of Ed can be deleted to generate a truncation variant of Ed without an extracellular domain (Ed-ΔX). Ed contains a signal sequence at the amino-terminus of the extracellular domain responsible for determining the orientation of the protein during its insertion in the membrane. Ed-ΔX would therefore encode a variation of Ed without its extracellular domain except for the signal sequence.

Deletion of the extracellular domain might result in the improper processing of the transgenic protein. As an alternative to the deletion construct, Ed-ΔX, a transgene encoding a chimeric Ed protein could also be utilized. Chimera using the extracellular domain of the receptor tyrosine kinase Torso and the intracellular domain of βPS-

integrin have been generated and successfully expressed to analyze the adhesion independent function of integrins (Martin-Bermudo and Brown, 1999). The same approach could therefore be used to generate a Torso:Ed fusion protein. It is important to note that the oligomerization of adhesion molecules such as cadherins and integrins is essential to their function (Halbleib and Nelson, 2006; Martin-Bermudo and Brown, 1999). This oligomerization is sometimes promoted by the extracellular domain and therefore if the oligomerization of Ed is essential and is promoted by the extracellular domain of Ed, neither of the Ed-ΔX nor the Torso:Ed chimera would be active. A point mutation in the extracellular domain of Torso results in the constitutive oligomerization of Torso ($Torso^{CO}$). Therefore, in parallel, a second version of the Torso:Ed, $Torso^{CO}$:Ed, chimera using the mutated version of Torso could be generated and its effect analyzed in parallel with the Ed-ΔX and Torso-Ed constructs.

To determine the function of the extracellular domain, Ed-ΔX, Torso:Ed and $Torso^{CO}$:Ed would be separately expressed in the epidermis of $ed^{M/Z}$ mutant embryos. In parallel, the full-length Ed transgene (Ed-Full) would be expressed in the epidermis of $ed^{M/Z}$ mutant embryos. The distribution of the constructs would be determined by antibody staining and their ability to rescue the $ed^{M/Z}$ lack of actomyosin cable would also be analyzed. Based on my previous findings, I would expect that only the expression of Ed-Full in the epidermis would rescue the formation of the actomyosin cable in $ed^{M/Z}$ embryos. I predict that the expression of either Ed-ΔX, Torso:Ed or $Torso^{CO}$:Ed in the epidermis of $ed^{M/Z}$ embryos would result in the uniform distribution of the transgenic protein around the cortex of the dorsal-most epidermal cells, and

therefore in the lack of an actomyosin cable. Finally, the expression of Ed-ΔX, Torso:Ed or Torso^{CO}:Ed in the epidermis of wild type embryos can identify whether maintaining the cytoplasmic tail at the leading edge during dorsal closure can prevent the formation of the actomyosin cable. According to my previous results in Ed-LE embryos, I would expect that maintaining the cytoplasmic tail of Ed at the leading edge will prevent the formation of the actomyosin cable similarly to the maintaining the full-length endogenous protein in Ed-LE embryos.

4.6.2 The cytoplasmic tail of Ed contains an unknown functional motif or motifs

The cytoplasmic tail of Ed contains only one known motif, the PDZ domain binding motif. The PDZ domain binding motif is dispensable for the function of Ed in the formation of an actomyosin cable. However, the cytoplasmic tail is essential for the formation of actomyosin cables suggesting that at least one other functional motif lies in the cytoplasmic tail of Ed besides the PDZ domain binding motif (section 3.4.8). What other functional domains are hidden in the approximately 300 amino acid-long cytoplasmic tail? Protein domain and motif prediction algorithm have failed to identify any known sequences in the cytoplasmic tail of Ed (C.L. unpublished results). An alternative method to identifying functional sequences is to align the primary sequence of the cytoplasmic tail of Ed from *Drosophila melanogaster* to that of the other sequenced *Drosophilia* genomes, the honeybee (*Apis mellifera*) and the mosquito (*Anopheles gambiae*). Regions with a high percentage of similarity between the sequences were conserved during evolution and therefore are more likely to be

functionally important. These regions can then be used as candidate sequences for the generation of Ed transgenes with truncations in the cytoplasmic tail. The functionality of the resulting transgenes can be tested in MARCM clones (as described in section 3.4.8). The motif responsible for the function of Ed in generating an actomyosin cable can then be utilized to identify interaction proteins via biochemical methods.

4.7 Conclusion

The development of multicellular organisms involves the rearrangements of flat sheets of epithelial cells into three-dimensional organs and gives the final shape of the organism. During these morphogenetic events, cells across a tissue coordinate local changes in their actin cytoskeleton to provide a synchronized and directional movement. For instance, during epithelial sheet movements, cells polarize their cytoplasm across the plane of the tissue and determine the leading edge of the migrating front by re-distributing actin regulators and concentrating them according to the direction of the movement. The local elaboration of different actin-based structures thus provides the driving force for directed cell movements. Contractile multicellular actomyosin cables comprise one such structure and are involved in morphogenesis during development while their assembly in mature adult epithelia participates in processes such as wound healing. During my Ph.D. work, I identified a role for the cell adhesion molecule Ed in the assembly of contractile actomyosin cables. Such structures contribute to morphogenetic events that occur during the closure of epidermal gaps during embryogenesis and the closure of appendage secreting tubes during oogenesis. During those morphogenetic events, groups of cells downregulate their expression of Ed and

therefore generate borders of differential expression with neighbouring Ed expressing tissues. Such interfaces result in the asymmetric distribution of Ed in the Ed expressing cells neighbouring non-expressing cells. From my research, we know that the asymmetric distribution of Ed around the cortex of a cell establishes the planar polarity of the actin cytoskeleton and thereby contributes to the formation of the actomyosin cable at the leading edge of the migration front.

To date, a functional homologue of Ed in higher organisms still remains to be identified. Yet, I suspect that the mechanism of Ed function is conserved via the function of another molecule or molecules. Therefore this local modulation of the cytoskeleton at differential Ed expression interfaces may represent a general mechanism for promoting epithelial morphogenesis.

Bibliography

Adelstein, R. S., Conti, M. A., Hathaway, D. R. and Klee, C. B. (1978). Phosphorylation of smooth muscle myosin light chain kinase by the catalytic subunit of adenosine 3': 5'-monophosphate-dependent protein kinase. *J Biol Chem* **253**, 8347-50.

Afshar, K., Stuart, B. and Wasserman, S. A. (2000). Functional analysis of the *Drosophila* diaphanous FH protein in early embryonic development. *Development* **127**, 1887-97.

Ahmed, A., Chandra, S., Magarinos, M. and Vaessin, H. (2003). Echinoid mutants exhibit neurogenic phenotypes and show synergistic interactions with the Notch signaling pathway. *Development* **130**, 6295-304.

Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y. and Kaibuchi, K. (1996). Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). *J Biol Chem* **271**, 20246-9.

Atkey, M. R., Lachance, J. F., Walczak, M., Rebello, T. and Nilson, L. A. (2006). Capicua regulates follicle cell fate in the *Drosophila* ovary through repression of mirror. *Development* **133**, 2115-23.

Bai, J., Chiu, W., Wang, J., Tzeng, T., Perrimon, N. and Hsu, J. (2001). The cell adhesion molecule Echinoid defines a new pathway that antagonizes the *Drosophila* EGF receptor signaling pathway. *Development* **128**, 591-601.

Barzik, M., Kotova, T. I., Higgs, H. N., Hazelwood, L., Hanein, D., Gertler, F. B. and Schafer, D. A. (2005). Ena/VASP proteins enhance actin polymerization in the presence of barbed end capping proteins. *J Biol Chem* **280**, 28653-62.

Bayer, C. A., Holley, B. and Fristrom, J. W. (1996). A switch in broad-complex zinc-finger isoform expression is regulated posttranscriptionally during the metamorphosis of *Drosophila* imaginal discs. *Dev Biol* **177**, 1-14.

Bear, J. E., Loureiro, J. J., Libova, I., Fassler, R., Wehland, J. and Gertler, F. B. (2000). Negative regulation of fibroblast motility by Ena/VASP proteins. *Cell* **101**, 717-28.

Bear, J. E., Svitkina, T. M., Krause, M., Schafer, D. A., Loureiro, J. J., Strasser, G. A., Maly, I. V., Chaga, O. Y., Cooper, J. A., Borisy, G. G. et al. (2002). Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. *Cell* **109**, 509-21.

Bement, W. M. (2002). Actomyosin rings: the riddle of the sphincter. *Curr Biol* **12**, R12-4.

Berg, C. A. (2005). The *Drosophila* shell game: patterning genes and morphological change. *Trends Genet* **21**, 346-55.

Berger, J., Suzuki, T., Senti, K. A., Stubbs, J., Schaffner, G. and Dickson, B. J. (2001). Genetic mapping with SNP markers in *Drosophila*. *Nat Genet* **29**, 475-81.

Bertet, C., Sulak, L. and Lecuit, T. (2004). Myosin-dependent junction remodelling controls planar cell intercalation and axis elongation. *Nature* **429**, 667-71.

Bloor, J. W. and Kiehart, D. P. (2002). *Drosophila* RhoA regulates the cytoskeleton and cell-cell adhesion in the developing epidermis. *Development* **129**, 3173-83.

Boettner, B., Harjes, P., Ishimaru, S., Heke, M., Fan, H. Q., Qin, Y., Van Aelst, L. and Gaul, U. (2003). The AF-6 homolog canoe acts as a Rap1 effector during dorsal closure of the *Drosophila* embryo. *Genetics* **165**, 159-69.

Braga, V. M., Del Maschio, A., Machesky, L. and Dejana, E. (1999). Regulation of cadherin function by Rho and Rac: modulation by junction maturation and cellular context. *Mol Biol Cell* **10**, 9-22.

Braga, V. M., Machesky, L. M., Hall, A. and Hotchin, N. A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J Cell Biol* **137**, 1421-31.

Buchsbaum, R. J. (2007). Rho activation at a glance. *J Cell Sci* **120**, 1149-52.

Carlier, M. F., Ducruix, A. and Pantaloni, D. (1999). Signalling to actin: the Cdc42-N-WASP-Arp2/3 connection. *Chem Biol* **6**, R235-40.

Carthew, R. W. (2005). Adhesion proteins and the control of cell shape. *Curr Opin Genet Dev* **15**, 358-63.

Chhabra, E. S. and Higgs, H. N. (2007). The many faces of actin: matching assembly factors with cellular structures. *Nat Cell Biol* **9**, 1110-21.

Clark, K., Langeslag, M., Figdor, C. G. and van Leeuwen, F. N. (2007). Myosin II and mechanotransduction: a balancing act. *Trends Cell Biol* **17**, 178-86.

Cohen, L. H., Pieterman, E., van Leeuwen, R. E., Overhand, M., Burm, B. E., van der Marel, G. A. and van Boom, J. H. (2000). Inhibitors of prenylation of Ras and other G-proteins and their application as therapeutics. *Biochem Pharmacol* **60**, 1061-8.

Cooke, R. (1975a). The bound nucleotide of actin. *J Supramol Struct* **3**, 146-53.

Cooke, R. (1975b). The role of the bound nucleotide in the polymerization of actin. *Biochemistry* **14**, 3250-6.

Dahmann, C. and Basler, K. (1999). Compartment boundaries: at the edge of development. *Trends Genet* **15**, 320-6.

Danjo, Y. and Gipson, I. K. (1998). Actin 'purse string' filaments are anchored by E-cadherin-mediated adherens junctions at the leading edge of the epithelial wound, providing coordinated cell movement. *J Cell Sci* **111 (Pt 22)**, 3323-32.

Darenfed, H. and Mandato, C. A. (2005). Wound-induced contractile ring: a model for cytokinesis. *Biochem Cell Biol* **83**, 711-20.

de Belle, J. S., Sokolowski, M. B. and Hilliker, A. J. (1993). Genetic analysis of the foraging microregion of *Drosophila* melanogaster. *Genome* **36**, 94-101.

Deng, W. M. and Bownes, M. (1997). Two signalling pathways specify localised expression of the Broad-Complex in *Drosophila* eggshell patterning and morphogenesis. *Development* **124**, 4639-47.

DiBello, P. R., Withers, D. A., Bayer, C. A., Fristrom, J. W. and Guild, G. M. (1991). The *Drosophila* Broad-Complex encodes a family of related proteins containing zinc fingers. *Genetics* **129**, 385-97.

Dobens, L. L. and Raftery, L. A. (2000). Integration of epithelial patterning and morphogenesis in *Drosophila* ovarian follicle cells. *Dev Dyn* **218**, 80-93.

Dorman, J. B., James, K. E., Fraser, S. E., Kiehart, D. P. and Berg, C. A. (2004). bullwinkle is required for epithelial morphogenesis during *Drosophila* oogenesis. *Dev Biol* **267**, 320-41.

Drees, F., Pokutta, S., Yamada, S., Nelson, W. J. and Weis, W. I. (2005). Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. *Cell* **123**, 903-15.

Eaton, S., Auvinen, P., Luo, L., Jan, Y. N. and Simons, K. (1995). CDC42 and Rac1 control different actin-dependent processes in the *Drosophila* wing disc epithelium. *J Cell Biol* **131**, 151-64.

Eddinger, T. J. and Meer, D. P. (2007). Myosin II isoforms in smooth muscle: heterogeneity and function. *Am J Physiol Cell Physiol* **293**, C493-508.

Edwards, K. A., Demsky, M., Montague, R. A., Weymouth, N. and Kiehart, D. P. (1997). GFP-moesin illuminates actin cytoskeleton dynamics in living tissue and demonstrates cell shape changes during morphogenesis in *Drosophila*. *Dev Biol* **191**, 103-17.

Egile, C., Loisel, T. P., Laurent, V., Li, R., Pantaloni, D., Sansonetti, P. J. and Carlier, M. F. (1999). Activation of the CDC42 effector N-WASP by the Shigella flexneri IcsA protein promotes actin nucleation by Arp2/3 complex and bacterial actin-based motility. *J Cell Biol* **146**, 1319-32.

Escudero, L. M., Wei, S. Y., Chiu, W. H., Modolell, J. and Hsu, J. C. (2003). Echinoid synergizes with the Notch signaling pathway in *Drosophila* mesothorax bristle patterning. *Development* **130**, 6305-16.

Etienne-Manneville, S. and Hall, A. (2002). Rho GTPases in cell biology. *Nature* **420**, 629-35.

Fox, D. T. and Peifer, M. (2007). Abelson kinase (Abl) and RhoGEF2 regulate actin organization during cell constriction in *Drosophila*. *Development* **134**, 567-78.

Franke, J. D., Montague, R. A. and Kiehart, D. P. (2005). Nonmuscle myosin II generates forces that transmit tension and drive contraction in multiple tissues during dorsal closure. *Curr Biol* **15**, 2208-21.

Fujiwara, I., Takahashi, S., Tadakuma, H., Funatsu, T. and Ishiwata, S. (2002). Microscopic analysis of polymerization dynamics with individual actin filaments. *Nat Cell Biol* **4**, 666-73.

Fujiwara, I., Vavylonis, D. and Pollard, T. D. (2007). Polymerization kinetics of ADP- and ADP-Pi-actin determined by fluorescence microscopy. *Proc Natl Acad Sci U S A* **104**, 8827-32.

Furuse, M. and Tsukita, S. (2006). Claudins in occluding junctions of humans and flies. *Trends Cell Biol* **16**, 181-8.

Gates, J., Mahaffey, J. P., Rogers, S. L., Emerson, M., Rogers, E. M., Sottile, S. L., Van Vactor, D., Gertler, F. B. and Peifer, M. (2007). Enabled plays key roles in embryonic epithelial morphogenesis in *Drosophila*. *Development* **134**, 2027-39.

Genova, J. L., Jong, S., Camp, J. T. and Fehon, R. G. (2000). Functional analysis of Cdc42 in actin filament assembly, epithelial morphogenesis, and cell signaling during *Drosophila* development. *Dev Biol* **221**, 181-94.

Glise, B. and Noselli, S. (1997). Coupling of Jun amino-terminal kinase and Decapentaplegic signaling pathways in *Drosophila* morphogenesis. *Genes Dev* **11**, 1738-47.

Goode, B. L. and Eck, M. J. (2007). Mechanism and function of formins in the control of actin assembly. *Annu Rev Biochem* **76**, 593-627.

Grawe, F., Wodarz, A., Lee, B., Knust, E. and Skaer, H. (1996). The *Drosophila* genes crumbs and stardust are involved in the biogenesis of adherens junctions. *Development* **122**, 951-9.

Grosshans, J., Wenzl, C., Herz, H. M., Bartoszewski, S., Schnorrer, F., Vogt, N., Schwarz, H. and Muller, H. A. (2005). RhoGEF2 and the formin Dia control the formation of the furrow canal by directed actin assembly during *Drosophila* cellularisation. *Development* **132**, 1009-20.

Halbleib, J. M. and Nelson, W. J. (2006). Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. *Genes Dev* **20**, 3199-214.

Harden, N. (2002). Signaling pathways directing the movement and fusion of epithelial sheets: lessons from dorsal closure in *Drosophila*. *Differentiation* **70**, 181-203.

Harden, N., Loh, H. Y., Chia, W. and Lim, L. (1995). A dominant inhibitory version of the small GTP-binding protein Rac disrupts cytoskeletal structures and inhibits developmental cell shape changes in *Drosophila*. *Development* **121**, 903-14.

Harden, N., Ricos, M., Ong, Y. M., Chia, W. and Lim, L. (1999). Participation of small GTPases in dorsal closure of the *Drosophila* embryo: distinct roles for Rho subfamily proteins in epithelial morphogenesis. *J Cell Sci* **112 (Pt 3)**, 273-84.

Harris, T. J. and Peifer, M. (2005). The positioning and segregation of apical cues during epithelial polarity establishment in *Drosophila*. *J Cell Biol* **170**, 813-23.

Hawley, R. J. and Waring, G. L. (1988). Cloning and analysis of the dec-1 female-sterile locus, a gene required for proper assembly of the *Drosophila* eggshell. *Genes Dev* **2**, 341-9.

Hickson, G. R., Echard, A. and O'Farrell, P. H. (2006). Rho-kinase controls cell shape changes during cytokinesis. *Curr Biol* **16**, 359-70.

Hinck, L., Nathke, I. S., Papkoff, J. and Nelson, W. J. (1994). Dynamics of cadherin/catenin complex formation: novel protein interactions and pathways of complex assembly. *J Cell Biol* **125**, 1327-40.

Homem, C. C. and Peifer, M. (2008). Diaphanous regulates myosin and adherens junctions to control cell contractility and protrusive behavior during morphogenesis. *Development* **135**, 1005-18.

Horne-Badovinac, S. and Bilder, D. (2005). Mass transit: epithelial morphogenesis in the *Drosophila* egg chamber. *Dev Dyn* **232**, 559-74.

Hoskins, R. A., Phan, A. C., Naeemuddin, M., Mapa, F. A., Ruddy, D. A., Ryan, J. J., Young, L. M., Wells, T., Kopczynski, C. and Ellis, M. C. (2001). Single nucleotide polymorphism markers for genetic mapping in *Drosophila* melanogaster. *Genome Res* **11**, 1100-13.

Hutson, M. S., Tokutake, Y., Chang, M. S., Bloor, J. W., Venakides, S., Kiehart, D. P. and Edwards, G. S. (2003). Forces for morphogenesis investigated with laser microsurgery and quantitative modeling. *Science* **300**, 145-9.

Hutterer, A., Betschinger, J., Petronczki, M. and Knoblich, J. A. (2004). Sequential roles of Cdc42, Par-6, aPKC, and Lgl in the establishment of epithelial polarity during *Drosophila* embryogenesis. *Dev Cell* **6**, 845-54.

Ip, Y. T., Kraut, R., Levine, M. and Rushlow, C. A. (1991). The dorsal morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in *Drosophila*. *Cell* **64**, 439-46.

Ip, Y. T., Park, R. E., Kosman, D., Bier, E. and Levine, M. (1992). The dorsal gradient morphogen regulates stripes of rhomboid expression in the presumptive neuroectoderm of the *Drosophila* embryo. *Genes Dev* **6**, 1728-39.

Islam, R., Wei, S. Y., Chiu, W. H., Hortsch, M. and Hsu, J. C. (2003). Neuroglian activates Echinoid to antagonize the *Drosophila* EGF receptor signaling pathway. *Development* **130**, 2051-9.

Jacinto, A., Wood, W., Balayo, T., Turmaine, M., Martinez-Arias, A. and Martin, P. (2000). Dynamic actin-based epithelial adhesion and cell matching during *Drosophila* dorsal closure. *Curr Biol* **10**, 1420-6.

Jacinto, A., Woolner, S. and Martin, P. (2002). Dynamic analysis of dorsal closure in *Drosophila*: from genetics to cell biology. *Dev Cell* **3**, 9-19.

Jaffe, A. B. and Hall, A. (2005). Rho GTPases: biochemistry and biology. *Annu Rev Cell Dev Biol* **21**, 247-69.

Jankovics, F. and Brunner, D. (2006). Transiently reorganized microtubules are essential for zippering during dorsal closure in *Drosophila* melanogaster. *Dev Cell* **11**, 375-85.

Jasper, H., Benes, V., Schwager, C., Sauer, S., Clauder-Munster, S., Ansorge, W. and Bohmann, D. (2001). The genomic response of the *Drosophila* embryo to JNK signaling. *Dev Cell* **1**, 579-86.

Jiang, J., Kosman, D., Ip, Y. T. and Levine, M. (1991). The dorsal morphogen gradient regulates the mesoderm determinant twist in early *Drosophila* embryos. *Genes Dev* **5**, 1881-91.

Joberty, G., Petersen, C., Gao, L. and Macara, I. G. (2000). The cell-polarity protein Par6 links Par3 and atypical protein kinase C to Cdc42. *Nat Cell Biol* **2**, 531-9.

Johndrow, J. E., Magie, C. R. and Parkhurst, S. M. (2004). Rho GTPase function in flies: insights from a developmental and organismal perspective. *Biochem Cell Biol* **82**, 643-57.

Jordan, P. and Karess, R. (1997). Myosin light chain-activating phosphorylation sites are required for oogenesis in *Drosophila*. *J Cell Biol* **139**, 1805-19.

Kaltschmidt, J. A., Lawrence, N., Morel, V., Balayo, T., Fernandez, B. G., Pelissier, A., Jacinto, A. and Martinez Arias, A. (2002). Planar polarity and actin dynamics in the epidermis of *Drosophila*. *Nat Cell Biol* **4**, 937-44.

Karess, R. E., Chang, X. J., Edwards, K. A., Kulkarni, S., Aguilera, I. and Kiehart, D. P. (1991). The regulatory light chain of nonmuscle myosin is encoded by spaghetti-squash, a gene required for cytokinesis in *Drosophila*. *Cell* **65**, 1177-89.

Kerkhoff, E. (2006). Cellular functions of the Spir actin-nucleation factors. *Trends Cell Biol* **16**, 477-83.

Kiehart, D. P., Galbraith, C. G., Edwards, K. A., Rickoll, W. L. and Montague, R. A. (2000). Multiple forces contribute to cell sheet morphogenesis for dorsal closure in *Drosophila*. *J Cell Biol* **149**, 471-90.

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K. et al. (1996). Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273**, 245-8.

Kobielak, A. and Fuchs, E. (2004). Alpha-catenin: at the junction of intercellular adhesion and actin dynamics. *Nat Rev Mol Cell Biol* **5**, 614-25.

Kobielak, A., Pasolli, H. A. and Fuchs, E. (2004). Mammalian formin-1 participates in adherens junctions and polymerization of linear actin cables. *Nat Cell Biol* **6**, 21-30.

Kovacs, E. M., Ali, R. G., McCormack, A. J. and Yap, A. S. (2002a). E-cadherin homophilic ligation directly signals through Rac and phosphatidylinositol 3-kinase to regulate adhesive contacts. *J Biol Chem* **277**, 6708-18.

Kovacs, E. M., Goodwin, M., Ali, R. G., Paterson, A. D. and Yap, A. S. (2002b). Cadherin-directed actin assembly: E-cadherin physically associates with the Arp2/3 complex to direct actin assembly in nascent adhesive contacts. *Curr Biol* **12**, 379-82.

Laplante, C. and Nilson, L. A. (2006). Differential expression of the adhesion molecule Echinoid drives epithelial morphogenesis in *Drosophila*. *Development* **133**, 3255-64.

Larsen, C. W., Hirst, E., Alexandre, C. and Vincent, J. P. (2003). Segment boundary formation in *Drosophila* embryos. *Development* **130**, 5625-35.

Lawrence, P. A. (1997). Developmental biology. Straight and wiggly affinities. *Nature* **389**, 546-7.

Lawrence, P. A. and Struhl, G. (1996). Morphogens, compartments, and pattern: lessons from drosophila? *Cell* **85**, 951-61.

Lecuit, T. (2005). Cell adhesion: sorting out cell mixing with echinoid? *Curr Biol* **15**, R505-7.

Lecuit, T. and Lenne, P. F. (2007). Cell surface mechanics and the control of cell shape, tissue patterns and morphogenesis. *Nat Rev Mol Cell Biol* **8**, 633-44.

Lecuit, T. and Wieschaus, E. (2000). Polarized insertion of new membrane from a cytoplasmic reservoir during cleavage of the *Drosophila* embryo. *J Cell Biol* **150**, 849-60.

Lee, T., Winter, C., Marticke, S. S., Lee, A. and Luo, L. (2000). Essential roles of *Drosophila* RhoA in the regulation of neuroblast proliferation and dendritic but not axonal morphogenesis. *Neuron* **25**, 307-16.

Leptin, M. and Grunewald, B. (1990). Cell shape changes during gastrulation in *Drosophila*. *Development* **110**, 73-84.

Lin, D., Edwards, A. S., Fawcett, J. P., Mbamalu, G., Scott, J. D. and Pawson, T. (2000). A mammalian PAR-3-PAR-6 complex implicated in Cdc42/Rac1 and aPKC signalling and cell polarity. *Nat Cell Biol* **2**, 540-7.

Lin, H. P., Chen, H. M., Wei, S. Y., Chen, L. Y., Chang, L. H., Sun, Y. J., Huang, S. Y. and Hsu, J. C. (2007). Cell adhesion molecule Echinoid associates with unconventional myosin VI/Jaguar motor to regulate cell morphology during dorsal closure in *Drosophila*. *Dev Biol* **311**, 423-33.

Machesky, L. M., Mullins, R. D., Higgs, H. N., Kaiser, D. A., Blanchoin, L., May, R. C., Hall, M. E. and Pollard, T. D. (1999). Scar, a WASp-related protein, activates nucleation of actin filaments by the Arp2/3 complex. *Proc Natl Acad Sci U S A* **96**, 3739-44.

Magie, C. R., Meyer, M. R., Gorsuch, M. S. and Parkhurst, S. M. (1999). Mutations in the Rho1 small GTPase disrupt morphogenesis and segmentation during early *Drosophila* development. *Development* **126**, 5353-64.

Major, R. J. and Irvine, K. D. (2005). Influence of Notch on dorsoventral compartmentalization and actin organization in the *Drosophila* wing. *Development* **132**, 3823-33.

Mandato, C. A., Benink, H. A. and Bement, W. M. (1999). Microtubule-actomyosin interactions in cortial flow and cytokinesis. *Cell Motil Cytoskeleton* **45**, 87-92.

Mandato, C. A. and Bement, W. M. (2003). Actomyosin transports microtubules and microtubules control actomyosin recruitment during Xenopus oocyte wound healing. *Current Biol* **13**, 1096-105.

Martin, P. and Parkhurst, S. M. (2004). Parallels between tissue repair and embryo morphogenesis. *Development* **131**, 3021-34.

Martin, S. G., Dobi, K. C. and St Johnston, D. (2001). A rapid method to map mutations in *Drosophila*. *Genome Biol* **2**, RESEARCH0036.

Martin-Bermudo, M. D. and Brown, N. H. (1999). Uncoupling integrin adhesion and signaling: the betaPS cytoplasmic domain is sufficient to regulate gene expression in the *Drosophila* embryo. *Genes Dev* **13**, 729-39.

Martin-Blanco, E. (1997). Regulation of cell differentiation by the *Drosophila* Jun kinase cascade. *Curr Opin Genet Dev* **7**, 666-71.

Martin-Blanco, E., Gampel, A., Ring, J., Virdee, K., Kirov, N., Tolkovsky, A. M. and Martinez-Arias, A. (1998). puckered encodes a phosphatase that mediates a feedback loop regulating JNK activity during dorsal closure in *Drosophila*. *Genes Dev* **12**, 557-70.

Matsuo, T., Takahashi, K., Suzuki, E. and Yamamoto, D. (1999). The Canoe protein is necessary in adherens junctions for development of ommatidial architecture in the *Drosophila* compound eye. *Cell Tissue Res* **298**, 397-404.

Millard, T. H. and Martin, P. (2008). Dynamic analysis of filopodial interactions during the zippering phase of *Drosophila* dorsal closure. *Development* **135**, 621-6.

Mondal, M. S., Wang, Z., Seeds, A. M. and Rando, R. R. (2000). The specific binding of small molecule isoprenoids to rhoGDP dissociation inhibitor (rhoGDI). *Biochemistry* **39**, 406-12.

Muller, H. A. (2000). Genetic control of epithelial cell polarity: lessons from *Drosophila*. *Dev Dyn* **218**, 52-67.

Muller, H. A. and Wieschaus, E. (1996). armadillo, bazooka, and stardust are critical for early stages in formation of the zonula adherens and maintenance of the polarized blastoderm epithelium in *Drosophila*. *J Cell Biol* **134**, 149-63.

Munro, E. M. (2006). PAR proteins and the cytoskeleton: a marriage of equals. *Curr Opin Cell Biol* **18**, 86-94.

Nakagawa, M., Fukata, M., Yamaga, M., Itoh, N. and Kaibuchi, K. (2001). Recruitment and activation of Rac1 by the formation of E-cadherin-mediated cell-cell adhesion sites. *J Cell Sci* **114**, 1829-38.

Nakayama, M., Goto, T. M., Sugimoto, M., Nishimura, T., Shinagawa, T., Ohno, S., Amano, M. and Kaibuchi, K. (2008). Rho-kinase phosphorylates PAR-3 and disrupts PAR complex formation. *Dev Cell* **14**, 205-15.

Nilson, L. A. and Schupbach, T. (1998). Localized requirements for windbeutel and pipe reveal a dorsoventral prepattern within the follicular epithelium of the *Drosophila* ovary. *Cell* **93**, 253-62.

Nilson, L. A. and Schupbach, T. (1999). EGF receptor signaling in *Drosophila* oogenesis. *Curr Top Dev Biol* **44**, 203-43.

Nobes, C. D. and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* **81**, 53-62.

Noren, N. K., Niessen, C. M., Gumbiner, B. M. and Burridge, K. (2001). Cadherin engagement regulates Rho family GTPases. *J Biol Chem* **276**, 33305-8.

Noselli, S. and Agnes, F. (1999). Roles of the JNK signaling pathway in *Drosophila* morphogenesis. *Curr Opin Genet Dev* **9**, 466-72.

Oda, H., Uemura, T. and Takeichi, M. (1997). Phenotypic analysis of null mutants for DE-cadherin and Armadillo in *Drosophila* ovaries reveals distinct aspects of their functions in cell adhesion and cytoskeletal organization. *Genes Cells* **2**, 29-40.

Paavilainen, V. O., Bertling, E., Falck, S. and Lappalainen, P. (2004). Regulation of cytoskeletal dynamics by actin-monomer-binding proteins. *Trends Cell Biol* **14**, 386-94.

Parks, S. and Wieschaus, E. (1991). The *Drosophila* gastrulation gene concertina encodes a G alpha-like protein. *Cell* **64**, 447-58.

Peralta, X. G., Toyama, Y., Hutson, M. S., Montague, R., Venakides, S., Kiehart, D. P. and Edwards, G. S. (2007). Upregulation of forces and morphogenic asymmetries in dorsal closure during *Drosophila* development. *Biophys J* **92**, 2583-96.

Pilot, F., Philippe, J. M., Lemmers, C. and Lecuit, T. (2006). Spatial control of actin organization at adherens junctions by a synaptotagmin-like protein Btsz. *Nature* **442**, 580-4.

Plastino, J., Lelidis, I., Prost, J. and Sykes, C. (2004a). The effect of diffusion, depolymerization and nucleation promoting factors on actin gel growth. *Eur Biophys J* **33**, 310-20.

Plastino, J., Olivier, S. and Sykes, C. (2004b). Actin filaments align into hollow comets for rapid VASP-mediated propulsion. *Curr Biol* **14**, 1766-71.

Pokutta, S., Drees, F., Takai, Y., Nelson, W. J. and Weis, W. I. (2002). Biochemical and structural definition of the l-afadin- and actin-binding sites of alpha-catenin. *J Biol Chem* **277**, 18868-74.

Pokutta, S. and Weis, W. I. (2000). Structure of the dimerization and beta-catenin-binding region of alpha-catenin. *Mol Cell* **5**, 533-43.

Pollard, T. D. (1986). Rate constants for the reactions of ATP- and ADP-actin with the ends of actin filaments. *J Cell Biol* **103**, 2747-54.

Pollard, T. D. (2007). Regulation of actin filament assembly by Arp2/3 complex and formins. *Annu Rev Biophys Biomol Struct* **36**, 451-77.

Pollard, T. D., Blanchoin, L. and Mullins, R. D. (2000). Molecular mechanisms controlling actin filament dynamics in nonmuscle cells. *Annu Rev Biophys Biomol Struct* **29**, 545-76.

Pollard, T. D. and Borisy, G. G. (2003). Cellular motility driven by assembly and disassembly of actin filaments. *Cell* **112**, 453-65.

Qiu, R. G., Abo, A. and Steven Martin, G. (2000). A human homolog of the C. elegans polarity determinant Par-6 links Rac and Cdc42 to PKCzeta signaling and cell transformation. *Curr Biol* **10**, 697-707.

Raftery, L. A. and Sutherland, D. J. (2003). Gradients and thresholds: BMP response gradients unveiled in *Drosophila* embryos. *Trends Genet* **19**, 701-8.

Rawlins, E. L., White, N. M. and Jarman, A. P. (2003). Echinoid limits R8 photoreceptor specification by inhibiting inappropriate EGF receptor signalling within R8 equivalence groups. *Development* **130**, 3715-24.

Ray, R. P., Arora, K., Nusslein-Volhard, C. and Gelbart, W. M. (1991). The control of cell fate along the dorsal-ventral axis of the *Drosophila* embryo. *Development* **113**, 35-54.

Reed, B. H., Wilk, R. and Lipshitz, H. D. (2001). Downregulation of Jun kinase signaling in the amnioserosa is essential for dorsal closure of the *Drosophila* embryo. *Curr Biol* **11**, 1098-108.

Ricos, M. G., Harden, N., Sem, K. P., Lim, L. and Chia, W. (1999). Dcdc42 acts in TGF-beta signaling during *Drosophila* morphogenesis: distinct roles for the Drac1/JNK and Dcdc42/TGF-beta cascades in cytoskeletal regulation. *J Cell Sci* **112 (Pt 8)**, 1225-35.

Ridley, A. J. and Hall, A. (1992). The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. *Cell* **70**, 389-99.

Ridley, A. J., Paterson, H. F., Johnston, C. L., Diekmann, D. and Hall, A. (1992). The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell* **70**, 401-10.

Riesgo-Escovar, J. R. and Hafen, E. (1997). *Drosophila* Jun kinase regulates expression of decapentaplegic via the ETS-domain protein Aop and the AP-1 transcription factor DJun during dorsal closure. *Genes Dev* **11**, 1717-27.

Rimm, D. L., Koslov, E. R., Kebriaei, P., Cianci, C. D. and Morrow, J. S. (1995). Alpha 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proc Natl Acad Sci U S A* **92**, 8813-7.

Rogers, S. L., Wiedemann, U., Hacker, U., Turck, C. and Vale, R. D. (2004). *Drosophila* RhoGEF2 associates with microtubule plus ends in an EB1-dependent manner. *Curr Biol* **14**, 1827-33.

Rohatgi, R., Ma, L., Miki, H., Lopez, M., Kirchhausen, T., Takenawa, T. and Kirschner, M. W. (1999). The interaction between N-WASP and the Arp2/3 complex links Cdc42-dependent signals to actin assembly. *Cell* **97**, 221-31.

Rosenblatt, J., Raff, M. C. and Cramer, L. P. (2001). An epithelial cell destined for apoptosis signals its neighbors to extrude it by an actin- and myosin-dependent mechanism. *Curr Biol* **11**, 1847-57.

Roth, S. (2003). The origin of dorsoventral polarity in *Drosophila*. *Philos Trans R Soc Lond B Biol Sci* **358**, 1317-29; discussion 1329.

Sahai, E. and Marshall, C. J. (2002). ROCK and Dia have opposing effects on adherens junctions downstream of Rho. *Nat Cell Biol* **4**, 408-15.

Samarin, S., Romero, S., Kocks, C., Didry, D., Pantaloni, D. and Carlier, M. F. (2003). How VASP enhances actin-based motility. *J Cell Biol* **163**, 131-42.

Sasaki, N., Miki, H. and Takenawa, T. (2000). Arp2/3 complex-independent actin regulatory function of WAVE. *Biochem Biophys Res Commun* **272**, 386-90.

Schock, F. and Perrimon, N. (2002a). Cellular processes associated with germ band retraction in *Drosophila*. *Dev Biol* **248**, 29-39.

Schock, F. and Perrimon, N. (2002b). Molecular mechanisms of epithelial morphogenesis. *Annu Rev Cell Dev Biol* **18**, 463-93.

Sellers, J. R. (1991). Regulation of cytoplasmic and smooth muscle myosin. *Curr Opin Cell Biol* **3**, 98-104.

Sept, D. and McCammon, J. A. (2001). Thermodynamics and kinetics of actin filament nucleation. *Biophys J* **81**, 667-74.

Sordella, R. and Van Aelst, L. (2008). Dialogue between RhoA/ROCK and members of the Par complex in cell polarity. *Dev Cell* **14**, 150-2.

Spencer, S. A. and Cagan, R. L. (2003). Echinoid is essential for regulation of Egfr signaling and R8 formation during *Drosophila* eye development. *Development* **130**, 3725-33.

Spradling, A. C. (1993). Developmental genetics of oogenesis. In *The Development of Drosophila melanogaster*, vol. 1 (ed. M. B. a. A. Martinez-Arias), pp. 1-70. Plainview: Cold Spring Harbor Laboratory Press.

Stathopoulos, A. and Levine, M. (2002). Dorsal gradient networks in the *Drosophila* embryo. *Dev Biol* **246**, 57-67.

Stronach, B. E. and Perrimon, N. (2001). Investigation of leading edge formation at the interface of amnioserosa and dorsal ectoderm in the *Drosophila* embryo. *Development* **128**, 2905-13.

Suzuki, A. and Ohno, S. (2006). The PAR-aPKC system: lessons in polarity. *J Cell Sci* **119**, 979-87.

Swan, L. E., Schmidt, M., Schwarz, T., Ponimaskin, E., Prange, U., Boeckers, T., Thomas, U. and Sigrist, S. J. (2006). Complex interaction of *Drosophila* GRIP PDZ domains and Echinoid during muscle morphogenesis. *Embo J* **25**, 3640-51.

Takahashi, K., Matsuo, T., Katsube, T., Ueda, R. and Yamamoto, D. (1998). Direct binding between two PDZ domain proteins Canoe and ZO-1 and their roles in regulation of the jun N-terminal kinase pathway in *Drosophila* morphogenesis. *Mech Dev* **78**, 97-111.

Takai, Y. and Nakanishi, H. (2003). Nectin and afadin: novel organizers of intercellular junctions. *J Cell Sci* **116**, 17-27.

Takaishi, K., Sasaki, T., Kotani, H., Nishioka, H. and Takai, Y. (1997). Regulation of cell-cell adhesion by rac and rho small G proteins in MDCK cells. *J Cell Biol* **139**, 1047-59.

Takenawa, T. and Miki, H. (2001). WASP and WAVE family proteins: key molecules for rapid rearrangement of cortical actin filaments and cell movement. *J Cell Sci* **114**, 1801-9.

Tanentzapf, G., Smith, C., McGlade, J. and Tepass, U. (2000). Apical, lateral, and basal polarization cues contribute to the development of the follicular epithelium during *Drosophila* oogenesis. *J Cell Biol* **151**, 891-904.

Tepass, U. (1996). Crumbs, a component of the apical membrane, is required for zonula adherens formation in primary epithelia of *Drosophila*. *Dev Biol* **177**, 217-25.

Tepass, U. (2002). Adherens junctions: new insight into assembly, modulation and function. *Bioessays* **24**, 690-5.

Tepass, U., Godt, D. and Winklbauer, R. (2002). Cell sorting in animal development: signalling and adhesive mechanisms in the formation of tissue boundaries. *Curr Opin Genet Dev* **12**, 572-82.

Tepass, U. and Hartenstein, V. (1994). The development of cellular junctions in the *Drosophila* embryo. *Dev Biol* **161**, 563-96.

Tepass, U., Tanentzapf, G., Ward, R. and Fehon, R. (2001). Epithelial cell polarity and cell junctions in *Drosophila*. *Annu Rev Genet* **35**, 747-84.

Trichet, L., Sykes, C. and Plastino, J. (2008). Relaxing the actin cytoskeleton for adhesion and movement with Ena/VASP. *J Cell Biol* **181**, 19-25.

Trybus, K. M. (1991). Regulation of smooth muscle myosin. *Cell Motil Cytoskeleton* **18**, 81-5.

Tzolovsky, G., Deng, W. M., Schlitt, T. and Bownes, M. (1999). The function of the broad-complex during *Drosophila* melanogaster oogenesis. *Genetics* **153**, 1371-83.

Van Aelst, L. and D'Souza-Schorey, C. (1997). Rho GTPases and signaling networks. *Genes Dev* **11**, 2295-322.

Van Aelst, L. and Symons, M. (2002). Role of Rho family GTPases in epithelial morphogenesis. *Genes Dev* **16**, 1032-54.

Van Buskirk, C. and Schupbach, T. (2002). Half pint regulates alternative splice site selection in *Drosophila*. *Dev Cell* **2**, 343-53.

Vasioukhin, V., Bauer, C., Yin, M. and Fuchs, E. (2000). Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell* **100**, 209-19.

Vasioukhin, V. and Fuchs, E. (2001). Actin dynamics and cell-cell adhesion in epithelia. *Curr Opin Cell Biol* **13**, 76-84.

Verma, S., Shewan, A. M., Scott, J. A., Helwani, F. M., den Elzen, N. R., Miki, H., Takenawa, T. and Yap, A. S. (2004). Arp2/3 activity is necessary for efficient formation of E-cadherin adhesive contacts. *J Biol Chem* **279**, 34062-70.

Wada, A., Kato, K., Uwo, M. F., Yonemura, S. and Hayashi, S. (2007). Specialized extraembryonic cells connect embryonic and extraembryonic epidermis in response to Dpp during dorsal closure in *Drosophila*. *Dev Biol* **301**, 340-9.

Ward, E. J. and Berg, C. A. (2005). Juxtaposition between two cell types is necessary for dorsal appendage tube formation. *Mech Dev* **122**, 241-55.

Ward, E. J., Zhou, X., Riddiford, L. M., Berg, C. A. and Ruohola-Baker, H. (2006). Border of Notch activity establishes a boundary between the two dorsal appendage tube cell types. *Dev Biol* **297**, 461-70.

Watanabe, N., Madaule, P., Reid, T., Ishizaki, T., Watanabe, G., Kakizuka, A., Saito, Y., Nakao, K., Jockusch, B. M. and Narumiya, S. (1997). p140mDia, a mammalian homolog of *Drosophila* diaphanous, is a target protein for Rho small GTPase and is a ligand for profilin. *Embo J* **16**, 3044-56.

Wei, S. Y., Escudero, L. M., Yu, F., Chang, L. H., Chen, L. Y., Ho, Y. H., Lin, C. M., Chou, C. S., Chia, W., Modolell, J. et al. (2005). Echinoid is a component of adherens junctions that cooperates with DE-Cadherin to mediate cell adhesion. *Dev Cell* **8**, 493-504.

Wieschaus, E., Audit, C. and Masson, M. (1981). A clonal analysis of the roles of somatic cells and germ line during oogenesis in *Drosophila*. *Dev Biol* **88**, 92-103.

Wieschaus, E. a. N.-V., C. (1986). Looking at embryos. In *Drosophila: A Practical Approach*, (ed. D. Roberts), pp. 199-228. Oxford: IRL Press.

Wodarz, A., Hinz, U., Engelbert, M. and Knust, E. (1995). Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of *Drosophila*. *Cell* **82**, 67-76.

Wodarz, A., Ramrath, A., Grimm, A. and Knust, E. (2000). *Drosophila* atypical protein kinase C associates with Bazooka and controls polarity of epithelia and neuroblasts. *J Cell Biol* **150**, 1361-74.

Wood, W., Jacinto, A., Grose, R., Woolner, S., Gale, J., Wilson, C. and Martin, P. (2002). Wound healing recapitulates morphogenesis in *Drosophila* embryos. *Nat Cell Biol* **4**, 907-12.

Woolner, S., Jacinto, A. and Martin, P. (2005). The small GTPase Rac plays multiple roles in epithelial sheet fusion--dynamic studies of *Drosophila* dorsal closure. *Dev Biol* **282**, 163-73.

Wu, J. Q., Kuhn, J. R., Kovar, D. R. and Pollard, T. D. (2003). Spatial and temporal pathway for assembly and constriction of the contractile ring in fission yeast cytokinesis. *Dev Cell* **5**, 723-34.

Xia, Y. and Karin, M. (2004). The control of cell motility and epithelial morphogenesis by Jun kinases. *Trends Cell Biol* **14**, 94-101.

Xu, T. and Rubin, G. M. (1993). Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development* **117**, 1223-37.

Yamada, S., Pokutta, S., Drees, F., Weis, W. I. and Nelson, W. J. (2005). Deconstructing the cadherin-catenin-actin complex. *Cell* **123**, 889-901.

Young, P. E., Richman, A. M., Ketchum, A. S. and Kiehart, D. P. (1993). Morphogenesis in *Drosophila* requires nonmuscle myosin heavy chain function. *Genes Dev* **7**, 29-41.

Zallen, J. A. and Wieschaus, E. (2004). Patterned gene expression directs bipolar planar polarity in *Drosophila*. *Dev Cell* **6**, 343-55.

Zhang, H. and Macara, I. G. (2008). The PAR-6 polarity protein regulates dendritic spine morphogenesis through p190 RhoGAP and the Rho GTPase. *Dev Cell* **14**, 216-26.

Appendix

McGill University

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

Projects involving potentially biohazardous materials should not be commenced without approval from Environmental Health & Safety. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: Laura Nilson PHONE: 398-6448

DEPARTMENT: Biology FAX: 398-5069

ADDRESS: Stewart Biology Building, Room N5/3 E-MAIL: laura.nilson@mcgill.ca

PROJECT TITLE:Epithelial morphogenesis in the Drosophila ovary.

---

2. EMERGENCY: Person(s) designated to handle emergencies

Name: Laura Nilson Phone No: work: 398-6448 home: 514-939-3302

Name: Caroline Laplante Phone No: work: 398-7488 home: 514-736-5090

---

3. FUNDING SOURCE OR AGENCY (specify):

Grant No.: RGPIN 238938-01 Beginning date: 04/2001 End date: 04/2006

---

4. Indicate if this is

☒ Renewal: procedures previously approved without alterations.

Approval End Date: April 1, 2006

☐ New funding source: project previously reviewed and approved under an application to another agency.

Agency: _____ Approval End Date: _____

☐ New project: project not previously reviewed.

☐ Approved project: change in biohazardous materials or procedures.

☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: Environmental Health & Safety approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in Health Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

Containment Level (select one): ☒ 1 ☐ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: _____ (SIGNATURE) date: 05 (day) 05 (month) 05 (year)

Approved by Environmental Health & Safety: d SA Louis (SIGNATURE) date: 05 (day) 05 (month) 05 (year)

Expiry: 30 (day) 04 (month) 06 (year)

*as defined in the "McGill Laboratory Biosafety Manual"

5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Trained in the safe use of biological safety cabinets within the last 3 years? If yes, indicate training date.
Laura Nilson	Biology	Assistant Professor	no
Caroline Laplante	Biology	Graduate Student	no
Aliaa Eleiche	Biology	Graduate Student	no
Jean-François Boisclair Lachance	Biology	Graduate Student	no

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

Drosophila DNA clones into E. coli plasmid and phage vectors (risk group 1)

ii) the procedures involving biohazards

Propagation of E. coli for preparation of cloned DNA. This procedure involves propagation on petri dishes of in liquid cultures of up to two liters.

iii) the protocol for decontaminating spills

For small spills, the material will be covered by paper toweling soaked in commercial bleach. Contaminated towels will be autoclaved in an autoclavable bag prior to disposal. The area will then be cleaned with 70% ethanol. For large spills (500 ml or more) the area will be evacuated, the door closed, and aerosols allowed to settle for 30 minutes. Clean-up will then proceed as above, with the assistance of the emergency response staff (x3000).

7. Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could increase the hazards?

o

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?
yes

9. What precautions will be taken to reduce production of infectious droplets and aerosols?
Regular examination of centrifuges and centrifuge tubes for defects; consistent balancing and frequent cleaning of centrifuges; use of mechanical pipetting aids when transferring biological substances.

10. Will the biohazardous materials in this project expose members of the research team to any risks that might require special training, vaccination or other protective measures? If yes, please explain.
no

11. Will this project produce combined hazardous waste – i.e. radioactive biohazardous waste, biohazardous animal carcasses contaminated with toxic chemicals, etc.? If yes, please explain how disposal will be handled.
no

12. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified

McGill University

APPLICATION TO USE BIOHAZARDOUS MATERIALS

Projects involving potentially biohazardous materials should not be commenced without approval from Environmental Health & Safety. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: Laura Nilson PHONE: 398-6448
DEPARTMENT: Biology FAX: 398-5069
ADDRESS: N3/5, Stewart Biology Building E-MAIL: laura.nilson@mcgill.ca

PROJECT TITLE(S):Regulation of epidermal growth factor mediated determination in the Drosophila ovary.

2. EMERGENCY: Person(s) designated to handle emergencies

Name: Laura Nilson Phone No: work: 398-6448 home: 514-939-3302
Name: Caroline Laplante Phone No: work: 398-7488 home: 514-736-5090

3. FUNDING SOURCE OR AGENCY: list all sources when information in Sections 5-12 is identical:

Source CIHR Grant No. 1G1-81645 Start date Oct 1, 2006 End date 9/30/2007
Source _____ Grant No. _____ Start date _____ End date _____
Source _____ Grant No. _____ Start date _____ End date _____

4. Indicate if this is

☐ Renewal: procedures previously approved without alterations.
Approval End Date: _____
☒ New funding source: project previously reviewed and approved under an application to another agency.
Agency: NCIC Approval End Date: 06/2005
☐ New project: project not previously reviewed.
☐ Approved project: change in biohazardous materials or procedures.
☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: Environmental Health & Safety approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the Public Health Agency of Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

Containment Level (select one): ☒ 1 ☐ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: _____ SIGNATURE date: 16 (day) 10 (month) 2006 (year)

Approved by Environmental Health & Safety: _____ SIGNATURE date: 16 (day) 10 (month) 06 (year)

Expiry: 30 (day) 09 (month) 07 (year)

5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Trained in the safe use of biological safety cabinets within the last 3 years? If yes, indicate training date.
Laura Nilson	Biology	Associate Professor	no
Jean-François Boisclair Lachance	Biology	Graduate Student	no
Lucia Caceres	Biology	Graduate Student	no

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

Drosophila DNA cloned in E. coli plasmid and phage vectors (risk group 1).

ii) the procedures involving biohazards

Propagation of E. coli for purification of cloned DNA. This procedure involves propagation on petri dishes or in liquid cultures of up to two litres.

iii) the protocol for decontaminating spills

For small spills, the material will be covered by paper toweling soaked in commercial bleach. Contaminated towels will be autoclaved in an autoclavable bag prior to disposal. The area will then be cleaned with 70% ethanol. For large spills (500 ml or more) the area will be evacuated, the door closed, and aerosols allowed to settle for 30 minutes. Clean-up will then proceed as above, with the assistance of the emergency response staff (x3000).

McGill University

APPLICATION TO USE BIOHAZARDOUS MATERIALS

Projects involving potentially biohazardous materials should not be commenced without approval from Environmental Health & Safety. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: Laura Nilson PHONE: 398-6448

DEPARTMENT: Biology FAX: 398-5069

ADDRESS: N3/5, Stewart Biology Building E-MAIL: laura.nilson@mcgill.ca

PROJECT TITLE(S):Regulation of epidermal growth factor mediated determination in the Drosophila ovary.

---

2. EMERGENCY: Person(s) designated to handle emergencies

Name: Laura Nilson Phone No: work: 398-6448 home: 514-939-3302

Name: Caroline Laplante Phone No: work: 398-7488 home: 514-736-5090

---

3. FUNDING SOURCE OR AGENCY: list all sources when information in Sections 5-12 is identical:

Source CIHR Grant No. IG1-81645 Start date Oct 1, 2006 End date 9/30/2007

Source _____ Grant No. _____ Start date _____ End date _____

Source _____ Grant No. _____ Start date _____ End date _____

---

4. Indicate if this is

☐ Renewal: procedures previously approved without alterations.

Approval End Date: _____

☒ New funding source: project previously reviewed and approved under an application to another agency.

Agency: NCIC Approval End Date: 06/2005

☐ New project: project not previously reviewed.

☐ Approved project: change in biohazardous materials or procedures.

☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: Environmental Health & Safety approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the Public Health Agency of Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

Containment Level (select one): ☒ 1 ☐ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: _____ (SIGNATURE) date: 16 (day) 10 (month) 2006 (year)

Approved by Environmental Health & Safety: _____ (SIGNATURE) date: _____ (day) _____ (month) _____ (year)

Expiry: _____ (day) _____ (month) _____ (year)

Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) that could increase the hazards?

8. Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?
yes

9. What precautions will be taken to reduce production of infectious droplets and aerosols?
Regular examination of centrifuges and centrifuge tubes for defects; consistent balancing and frequent cleaning of centrifuges; use of mechanical pipetting aids when transferring biological substances.

10. Will the biohazardous materials in this project expose members of the research team to any risks that might require special training, vaccination or other protective measures? If yes, please explain.
no

11. Will this project produce combined hazardous waste – i.e. radioactive biohazardous waste, biohazardous animal carcasses contaminated with toxic chemicals, etc.? If yes, please explain how disposal will be handled.
no

12. List the biological safety cabinets to be used.

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified

****
*** TX REPORT ***
****

TRANSMISSION OK

TX/RX NO	2550
CONNECTION TEL	4626
SUBADDRESS	
CONNECTION ID	
ST. TIME	10/16 15:59
USAGE T	01'42
PGS. SENT	5
RESULT	OK

DEPARTMENT OF BIOLOGY
MCGILL UNIVERSITY

FACSIMILE TRANSMITTAL SHEET

TO: YOLANDA MANCINI	FROM: Laura Nilson
COMPANY: RGO	DATE: OCTOBER 16, 2006
FAX NUMBER: 398-4626	TOTAL NO. OF PAGES INCLUDING COVER: 5
PHONE NUMBER:	SENDER'S PHONE NUMBER: (514) 398-6448
RE: Biohazard Permit	SENDER'S EMAIL ADDRESS: laura.nilson@mcgill.ca

* URGENT * FOR REVIEW * PLEASE COMMENT * PLEASE REPLY * PLEASE RECYCLE

Thanks! Let me know if you need anything else.

Laura