Cellular Mechanisms of Embryonic Tissue Organization

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Biomaterials and Biomedical Engineering
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Abstract

During early embryonic development, cell and tissue movements must be carefully orchestrated to prevent developmental anomalies. Here, I characterize cellular dynamics during axis elongation, a conserved developmental process in which the head-to-tail (anterior-posterior) axis of an animal extends. In *Drosophila*, early stages of axis elongation are driven by cell intercalation in the ectoderm, in which cells exchange neighbours through the formation of transient multicellular vertices. Vertices are resolved by the systematic assembly of new cell interfaces parallel to the head-to-tail axis of the embryo. I show that new junctions elongate in pulses anti-correlated with the periodic contractions of the cells anterior and posterior to the new contact. Inhibiting actomyosin contractility disrupts both the rate and directionality of new junction assembly. Disrupting contractility in the cells anterior and posterior to the new edge disrupts vertex resolution and slows down new edge elongation, while preventing contraction of the dorsal and ventral cells mainly affects the maintenance and lengthening of the new cell interface. Hypercontraction of the cells anterior and posterior to the new edge accelerates the rate of new edge assembly. Finally, applying ectopic tension orthogonal to the characteristic orientation of vertex resolution is sufficient to
alter the direction of new edge formation, suggesting that local mechanical forces associated with actomyosin contractility direct the assembly of new cell contacts during multicellular vertex resolution during ectoderm extension. During later stages of axis elongation, a cell population on the underside of the embryo, the mesectoderm, undergoes oriented cell divisions to relieve strain generated from intercalating cells. Following mitoses, cell polarity is reversed in mesectoderm cells, resulting in the formation of supracellular myosin cables that segregate the mesectoderm from the ectoderm. Increased tension at the boundary prevents cell mixing and contributes to maintenance by stabilizing myosin. Mechanical and pharmacological cable disruption results in the premature internalization of the mesectoderm. Thus, our findings demonstrate that myosin cables can maintain tissue position while acting as boundaries that prevent cell mixing. Together, my work identifies two distinct mechanisms by which subcellular dynamics can translate into tissue-level organization during embryonic development.
Acknowledgements

These last six years have been exciting, difficult, fun, scary, and, at times, just plain excruciating… and beyond anything else, they have changed me. During this time, I have been overwhelmed with support from a number of people who are very special to me. I could never have believed in myself enough to do this without you!

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On to the next chapter =)
# Table of contents

Abstract .......................................................................................................................... i

Acknowledgements ........................................................................................................ iii

Table of contents ............................................................................................................ vi

List of figures ................................................................................................................... ix

List of equations ............................................................................................................. xi

List of other resources .................................................................................................. xii

Abbreviations ................................................................................................................ xiii

Chapter 1: Introduction .................................................................................................. 1
  1.1 Convergent extension shapes tissues during embryogenesis .............................. 1
  1.2 Molecular and mechanical asymmetries orient cell and tissue movements .......... 4
  1.3 Germband extension involves tissue-tissue interactions .................................... 8
  1.4 The ectoderm-mesectoderm interface during germband extension .................... 12
  1.5 Mechanisms of tissue boundary formation and maintenance ........................... 13
    1.5.1 Differences in gene expression establish compartment boundaries ............ 15
    1.5.2 Cell sorting through differential adhesion and cell repulsion ...................... 16
    1.5.3 Cytoskeletal contractility can resist cell mixing ........................................... 18
  1.6 Do mesectoderm fate and function depend on its position? .............................. 20
  1.7 Thesis overview and objectives ........................................................................... 21

Chapter 2: Local mechanical forces promote polarized junctional assembly and axis
  elongation in *Drosophila* ............................................................................................. 23
  2.1 Abstract ................................................................................................................. 24
  2.2 Introduction ............................................................................................................ 24
  2.3 Materials and Methods .......................................................................................... 27
    2.3.1 Fly stocks ...................................................................................................... 27
    2.3.2 Time-lapse imaging ..................................................................................... 27
    2.3.3 Laser ablation .............................................................................................. 27
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.2 Factors directing <em>de novo</em> junction formation during axis elongation</td>
<td>84</td>
</tr>
<tr>
<td>4.2.1 Tricellular vertices as a mediator of junctional assembly?</td>
<td>84</td>
</tr>
<tr>
<td>4.2.2 Mechanical feedback during cell intercalation</td>
<td>88</td>
</tr>
<tr>
<td>4.2.3 Molecular regulators of actin structures at nascent interfaces</td>
<td>92</td>
</tr>
<tr>
<td>4.3 Mechanisms underlying tissue boundary formation and maintenance during embryonic fly development</td>
<td>94</td>
</tr>
<tr>
<td>4.3.1 Organization of molecular polarity during tissue boundary formation</td>
<td>95</td>
</tr>
<tr>
<td>4.3.2 Maintenance of ME boundaries until tissue internalization</td>
<td>97</td>
</tr>
<tr>
<td>4.3.3 Is differential adhesion a feature of the ME boundary?</td>
<td>99</td>
</tr>
<tr>
<td>4.4 Conclusions</td>
<td>104</td>
</tr>
<tr>
<td>References</td>
<td>105</td>
</tr>
</tbody>
</table>

Appendix A: Quantitative modelling of epithelial morphogenesis: integrating cell mechanics and molecular dynamics ........................................... 135
  A.1 Abstract .................................................................................. 136
  A.2 Why computer models? Advantages and limitations ......................... 136
  A.3 Vertex models: a primer .............................................................. 138
  A.4 Modelling complex morphogenetic processes: dorsal closure ............. 142
  A.5 Conclusion .............................................................................. 154

Appendix B: Mechanical regulation of actin at resolving vertices ............ 156

Appendix C: Candidate screen for actin regulators .................................. 159
List of figures

Figure 1.1: Convergent extension shapes tissues during embryogenesis.

Figure 1.2: Cellular mechanisms of Drosophila germband extension.

Figure 1.3: Morphogenetic processes adjacent to Drosophila germband extension.

Figure 1.4: Compartment boundaries isolate epithelial regions to direct tissue-tissue interactions.

Figure 2.1: Axis elongation in Drosophila is driven by neighbour exchange events.

Figure 2.2: Directional assembly of new interfaces during vertex resolution is associated with pulsatile apical contractions and requires contractile activity.

Figure 2.3: Dorsal and ventral cells oscillate with new DV interfaces.

Figure 2.4: Directional assembly of new DV interfaces during vertex resolution requires actomyosin contractility.

Figure 2.5: Par complex localization is affected by Y-27632, but not by Cytochalasin D.

Figure 2.6: Oriented assembly of new DV interfaces requires actin-based contraction.

Figure 2.7: Resolving edges sustain increased mechanical tension during axis elongation.

Figure 2.8: The retraction velocity after ablation of new and control DV edges is not anti-correlated with their length.

Figure 2.9: New DV edges do not display a significant myosin accumulation.

Figure 2.10: Local actomyosin contractility is necessary for vertex resolution and new DV interface assembly.

Figure 2.11: Wounded cells undergo apical constriction and induce ectopic tension on adjacent cell-cell junctions.

Figure 2.12: Local mechanical tension is sufficient to promote and orient new interface assembly during vertex resolution.

Figure 3.1: Myosin, Bazooka, and Rok reverse their polarity after mesectoderm cell divisions.

Figure 3.2: ME cables are at least partially comprised of a mesectoderm-specific myosin population.

Figure 3.3: The ME boundary sustains increased tension and displays myosin stabilization.
Figure 3.4: Tension regulates myosin dynamics at the ME boundary and mesectoderm position. ................................................................. 68
Figure 3.5: Myosin density throughout the progression of mesectoderm internalization. .... 70
Figure 3.6: Y-27632 treatment disrupts the myosin cable at the ME boundary. ............... 71
Figure 3.7: Rok inhibition results in premature mesectoderm internalization. ............... 73
Figure 3.8: Rok inhibition results in upregulated protrusive activity. ......................... 75
Figure 4.1: TCJ rearrangement and cell geometry during neighbour exchange ............. 85
Figure 4.2: Homogenous myosin levels under a force balance at the ME boundary may contribute to its maintenance. ......................................................... 100
Figure 4.3: E-cadherin levels are reduced at the ME boundary. ................................. 102
Figure A1: Representing cells as nodes and edges. .................................................. 139
Figure A2: A simplified model of myosin minifilament assembly and disassembly .... 144
Figure A3: Dorsal closure in Drosophila embryos. ................................................... 147
Figure B1: The oscillatory assembly of new interfaces is positively correlated with the pulsatile accumulation of actin. ......................................................... 157
Figure B2: Mechanical tension promotes actin accumulation at resolving DV interfaces... 158
Figure C1: Diaphanous depletion results in defects in germband extension ............... 160
Figure C2: Slingshot, a Cofilin phosphatase, is necessary for early embryogenesis in Drosophila. ............................................................................... 161
List of equations

(2.1) rate of elongation \((t) = \frac{l(t) - l(t_0)}{t - t_0} \) .................................................................29

(3.1) \( f(t) = 100 \times \left( \frac{l_{ROI}(t) - l_{BG}(t)}{l_{ROI}(before) - l_{BG}(before)} \right) \times \left( \frac{l_{ROI}(0) - l_{BG}(0)}{l(0) - l_{BG}(before)} \right) \) ...............................................................57

(3.2) rate of change in myosin fluorescence (%) = 100\% \times (m(t) - m(t_0)) ..................................................59

(3.3) rate of change in mesectoderm width (%) = 100\% \times (w(t) - w(t_0)) ..................................................59

(A.1) \( F_i = \mu \frac{dr_i}{dt} \) ..................................................................................................................140

(A.2) \( U = \sum_{f or \ every \ cell, \alpha} \left( K_{\alpha} \left( A_{\alpha} - A_{a_0} \right)^2 + I_{\alpha} P_{\alpha}^2 \right) + \sum_{f or \ every \ junction, jk} \Lambda_{jk} L_{jk} \) .................141

(A.3) \( \frac{dm_f(t)}{dt} = k_{a} R(t) m_c(t) - k_{d} m_f(t) \) ...................................................................................143

(A.4) \( \phi(x, t) = -D \frac{\partial c(x, t)}{\partial x} = -D \nabla c \) .................................................................................145

(A.5) \( \frac{\partial \phi(x, t)}{\partial x} = -\frac{\partial c(x, t)}{\partial t} \) or \( \nabla \phi = -\frac{\partial c}{\partial t} \) .................................................................................145

(A.6) \( \frac{\partial c(x, t)}{\partial t} = D \frac{\partial^2 c(x, t)}{\partial x^2} \) or \( \frac{\partial c}{\partial t} = D \nabla^2 c \) .................................................................................145

(A.7) \( \frac{\partial m_c(x, t)}{\partial t} = D \frac{\partial^2 m_c(x, t)}{\partial x^2} + k_{a} m_f(x, t) - k_{a} \rho(x, t) m_c(x, t) \) ........................................145
List of other resources

Box A1: Defining motion equations................................................................. 141
Box A2: Modelling “well-stirred” systems: ordinary differential equations........ 143
Box A3: Modelling with diffusion: partial differential equations....................... 145
Table C1: Candidate screen for actin regulators involved in early Drosophila embryonic development................................................................. 159
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABD</td>
<td>actin-binding domain</td>
</tr>
<tr>
<td>abl</td>
<td>Abelson kinase</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>AFM</td>
<td>atomic force microscopy</td>
</tr>
<tr>
<td>AP</td>
<td>anterior-posterior</td>
</tr>
<tr>
<td>aPKC</td>
<td>atypical protein kinase C</td>
</tr>
<tr>
<td>BCJ</td>
<td>bicellular junction</td>
</tr>
<tr>
<td>capu</td>
<td>Cappuccino</td>
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<tr>
<td>chic</td>
<td>Chickadee</td>
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<tr>
<td>cib</td>
<td>Ciboulot</td>
</tr>
<tr>
<td>DAAM</td>
<td>Dishevelled associated activator of morphogenesis</td>
</tr>
<tr>
<td>dia</td>
<td>Diaphanous</td>
</tr>
<tr>
<td>DV</td>
<td>dorsal-ventral</td>
</tr>
<tr>
<td>FALI</td>
<td>fluorophore-assisted laser inhibition</td>
</tr>
<tr>
<td>fhos</td>
<td>Formin homology 2 domain containing ortholog</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>form3</td>
<td>Formin3</td>
</tr>
<tr>
<td>frl</td>
<td>Formin-like</td>
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<tr>
<td>FRET</td>
<td>Förster resonance energy transfer</td>
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<tr>
<td>GEF</td>
<td>guanine exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>mCh</td>
<td>mCherry</td>
</tr>
<tr>
<td>ME</td>
<td>mesectoderm-ectoderm</td>
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<tr>
<td>PALM</td>
<td>photoactivated localization microscopy</td>
</tr>
<tr>
<td>qua</td>
<td>Quail</td>
</tr>
<tr>
<td>rok</td>
<td>Rho-kinase</td>
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<tr>
<td>s.d.</td>
<td>standard deviation</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>s.e.m.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SIESTA</td>
<td>Scientific Image Segmentation and Analysis</td>
</tr>
<tr>
<td>sim</td>
<td>Single-minded</td>
</tr>
<tr>
<td>sqh</td>
<td>Spaghetti squash (<em>Drosophila</em> myosin regulatory light chain)</td>
</tr>
<tr>
<td>ssh</td>
<td>Slingshot</td>
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<tr>
<td>SRRF</td>
<td>super-resolution radial fluctuation</td>
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<td>STORM</td>
<td>stochastic optical reconstruction microscopy</td>
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<tr>
<td>TCJ</td>
<td>tricellular junction</td>
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<tr>
<td>TFM</td>
<td>traction force microscopy</td>
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<tr>
<td>TRiP</td>
<td>Transgenic RNAi Project</td>
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<tr>
<td>UAS</td>
<td>upstream activating sequence</td>
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<td>UV</td>
<td>ultraviolet</td>
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Chapter 1: Introduction

1.1 Convergent extension shapes tissues during embryogenesis

Patterns and processes established during embryonic development ultimately determine tissue organization and shape. Thus, cell movement and coordination must be elaborately orchestrated in both time and space to ensure that the final form of a tissue is appropriately achieved. Often, tissue function depends heavily on its structure—for example, to maximize surface area available for gas exchange, lung epithelia undergo many rounds of tubule elongation and branching (Hsia et al., 2016; Schittny, 2017); to provide adequate structural integrity, bone must be appropriately dense and shaped according to body region (Sommerfeldt and Rubin, 2001; Grabowski, 2015); and for the heart to function in blood circulation, it must achieve a final structure capable of containing volumes of blood, and have the ability to pump blood to body extremities (Sedmera, 2011; Andres-Delgado and Mercader, 2016). Importantly, the final shape of a tissue does not arise by chance, as development proceeds with vigorous repeatability between animals, and many chemical, biological, mechanical, and physical cues are conserved across species to ensure that morphogenetic events proceed stereotypically. Developmental processes that occur abnormally can result in malformed tissues and organs with impeded function; indeed, nearly 8 million infants are born with birth defects each year worldwide (Christianson et al., 2006). Thus, uncovering the mechanisms that ensure robust development during embryogenesis will provide insight on how tissues achieve their final shape. Furthermore, this will also inform our current understanding of developmental disorders, and how to develop regenerative medicine and tissue engineering strategies to treat them.

A common tissue-shaping process occurs when a tissue undergoes shortening along one axis (convergence) while simultaneously lengthening along one or more perpendicular axes (extension) (Figure 1.1A). Convergent extension recurs throughout development and is highly conserved between species and organs, from the cochlea in the mouse (Yamamoto et al., 2009), to kidney tubules in frogs (Lienkamp et al., 2012), as well as the vertebrate neural
Figure 1.1: Convergent extension shapes tissues during embryogenesis.

(A) Convergent extension occurs when a tissue shortens along one axis and lengthens along one or more perpendicular axes. (B) Germband position at the beginning (left), during (centre), and at late stages of axis elongation (right). Arrows indicate the direction of cell movement. White arrowheads delimit the germband. Anterior left, dorsal top. Scale bar, 100 µm. Reprinted from eLife Sciences Publications, Ltd. from Yu, J.C. & Fernandez-Gonzalez, R. (2016). Local mechanical forces promote polarized junctional assembly and axis elongation in Drosophila. eLife, 5, e10757.
plate (Williams et al., 2014) and neural tube (Davidson and Keller, 1999). Central to convergence and extension are mechanisms that promote cell coordination, which enable cell movements without disrupting tissue integrity. Mechanical forces generated within cells by cytoskeletal networks and transmitted across cells by cell-cell and cell-extracellular matrix adhesion molecules can contribute to coordinating cell behaviours during tissue morphogenesis.

Axis elongation is one of the first conserved morphogenetic processes during embryonic development. During axis elongation, the head-to-tail (anterior-posterior, or AP) axis of the animal is established. Severe consequences can arise when axis elongation is impaired—premature axis truncation not only results in a shortened body axis, but can also have compounded deleterious effects later on in development. In the mouse, axis truncation is accompanied by misspecification of neural crest tissue, misdirected cell proliferation, and a delay in neural tube closure, with extended delays resulting in spina bifida (Anderson et al., 2016). Likewise, developmental defects in the chick embryo resulting from axis truncation can generate excess neural tissue at the expense of the mesoderm, which then becomes unable to form vertebrae and somites in the tail bud (Freese et al., 2014). Disruption of signalling pathways that are required for axis elongation in the mouse can furthermore result in shortened, kinked, or absence of tails; abnormal fusion, shortening/malformation, or absence of vertebrae; or complete developmental arrest (Wilson et al., 2009). Thus, proper regulation of axis elongation ensures that development can proceed stereotypically once an appropriate body plan has been established.

Convergence and extension drives axis elongation in the embryo of the fruit fly Drosophila melanogaster. In Drosophila, axis elongation occurs three hours post-fertilization, in an epithelial monolayer referred to as the germband (Irvine and Wieschaus, 1994), which, acting as the embryonic ectoderm, will give rise to the epidermis and the central nervous system (Hartenstein and Campos-Ortega, 1985). Over the course of approximately one hour, the germband narrows along its back-to-front (dorsal-ventral, or DV) axis, concomitant with its elongation along the AP axis (Figure 1.1B). The study of convergent extension during axis elongation in Drosophila allows for live imaging of the process, and can benefit from
the genetic tractability of the system, as well as its amenability to biophysical and pharmacological manipulations.

1.2 Molecular and mechanical asymmetries orient cell and tissue movements

Axis elongation in *Drosophila* is largely driven by cellular rearrangements referred to as cell intercalation, during which cells systematically exchange neighbours (Irvine and Wieschaus, 1994; Bertet et al., 2004). Cell intercalation is a fundamental, conserved cell behaviour that shapes tissues during development. In the *Drosophila* germband, cells intercalate through neighbour exchange events, in which a cell interface that separates an anterior and a posterior cell neighbour (AP interface) contracts, forming a vertex where four cells meet (Figure 1.2A, top). The vertex is resolved with the formation of a new interface (DV interface) separating the dorsal and ventral neighbours, which meet as a consequence of the contraction of the AP interface (Irvine and Wieschaus, 1994; Bertet et al., 2004; Blankenship et al., 2006; Fernandez-Gonzalez et al., 2009). Neighbour exchanges also occur in higher-order structures consisting of five or more cells, termed rosettes (Blankenship et al., 2006). Here, a series of aligned AP interfaces shared by 5 to 11 cells contract, forming a multicellular vertex that then resolves through the assembly of a series of new DV interfaces (Blankenship et al., 2006) (Figure 1.2A, bottom). Rosettes provide an efficient mechanism of convergence and extension, as the change in the aspect ratio of the cells involved is greater for rosettes than for neighbour exchange events involving only four cells (Blankenship et al., 2006). The recurrence of oriented neighbour exchange events throughout the germband leads to narrowing of the tissue along its DV axis and elongation along the AP axis. Together, neighbour exchange and rosette structures allow cells to coordinate convergent extension movements in a systematic and ordered manner.

The directionality of intercalary behaviours in the germband originates from maternally-deposited patterning gradients, which then direct the formation of striped signalling cues along the trunk of the embryo, including the pair rule genes Eve and Runt (Schupbach and Wieschaus, 1986; Nusslein-Volhard et al., 1987; St Johnston and Nusslein-Volhard, 1992;
Figure 1.2: Cellular mechanisms of *Drosophila* germband extension.

(A) In the lateral germband, cell intercalation consists of neighbour exchange events (inset, top) and rosettes of 5-11 cells (inset, bottom). AP interfaces (left, red) contract into multicellular vertex (middle, red), which resolves along the perpendicular axis through the assembly of a new DV junction(s) (right, red). (B) Cytoskeletal and adherens junction proteins are planar polarized in the germband. Rho-kinase, myosin, and actin (magenta) localize to AP junctions, while Bazooka, E-cadherin, and β-catenin (green) polarize to DV interfaces. (C) Regulators upstream of myosin contraction at AP interfaces. (D) Germband extension also has contributions from cell shape changes in the lateral germband (top), and oriented cell divisions at the mesectoderm (bottom) and the posterior tip of the germband. Yellow arrows indicate mesectoderm division angle, oriented along the AP axis of the embryo. Cell outlines are marked with Resille:GFP (top) and Gap43:mCherry (bottom). Scale bars, 5 μm (top) and 10 μm (bottom).
Irvine and Wieschaus, 1994), and their downstream effectors, the Toll family of receptors (Pare et al., 2014). The activity of all of these factors contribute towards the asymmetric distribution of key cytoskeletal and cell-cell adhesion proteins in the plane of the tissue, in a pattern referred to as planar polarity (Figure 1.2B). Actin is a cytoskeletal protein that forms filaments, which provide structure to the cell, but also act as substrates for motor proteins (Vicente-Manzanares et al., 2009). The motor protein non-muscle myosin II (hereafter referred to as myosin) can slide actin filaments with opposite orientation with respect to each other, generating contractile forces (Vicente-Manzanares et al., 2009). Actin and myosin are both planar polarized to AP junctions in the germband (Bertet et al., 2004; Zallen and Wieschaus, 2004; Blankenship et al., 2006). Upstream regulators of both myosin and actin have later been found to also be polarized at AP interfaces. The serine/threonine kinase Rho-kinase (Rok), a myosin activator, as well as the small GTPase Rho1 and the Rok-binding protein Shroom, all localize at AP junctions (Simoes et al., 2010; Simoes et al., 2014). Similarly, the junctional-cytoskeletal linker Canoe/Afadin (Sawyer et al., 2011) and the actin regulator Abelson kinase (Abl) (Tamada et al., 2012) also localize to AP interfaces. In contrast, the adherens junction components E-cadherin and Armadillo/β-catenin, and the cell polarity protein Bazooka/Par-3 preferentially localize to DV junctions (Zallen and Wieschaus, 2004; Blankenship et al., 2006; Simoes et al., 2010). Together, the planar polarity system in the germband establishes molecular asymmetries prior to extension that will ultimately orchestrate tissue movements.

The contraction of AP interfaces during neighbour exchange and rosette formation has been characterized in detail. Actomyosin enrichment drives interfacial contraction in a tension-dependent manner (Rauzi et al., 2008; Fernandez-Gonzalez et al., 2009). In addition to myosin association with cell-cell junctions, pulses of medial-apical myosin cause anisotropic oscillations of the apical cell surface, which promote the periodic ratchet-like contraction of AP interfaces (Rauzi et al., 2010; Fernandez-Gonzalez and Zallen, 2011; Sawyer et al., 2011; Levayer and Lecuit, 2013; Clement et al., 2017). Myosin asymmetry and activity are controlled by phosphorylation of two conserved residues, a serine and a threonine (Sellers et al., 1985; Jordan and Karess, 1997; Komatsu et al., 2000). The main activator of myosin is the kinase Rho-kinase, which can directly phosphorylate and activate myosin (Amano et al.,
1996), and deactivates the myosin phosphatase (Kimura et al., 1996). Rho-kinase, in turn, is regulated by the actin-associated protein Shroom (Simoes et al., 2014) and other components of the Rho pathway (Munjal et al., 2015), which also interacts with the guanine exchange factor RhoGEF2 (Levayer et al., 2011) and other factors associated with G-protein coupled receptors (Kerridge et al., 2016; Jha et al., 2018), all with downstream roles in cytoskeletal contractility (Figure 1.2C). Disrupting AP patterning abolishes actomyosin planar polarity and disrupts germband extension (Zallen and Wieschaus, 2004; Blankenship et al., 2006; Fernandez-Gonzalez et al., 2009), implicating AP patterning as key information that the germband requires for proper cytoskeletal polarity and cell intercalation.

Cell-cell junctions must be disassembled for cell interfaces to disappear. Germband cells are connected to each other through adherens junctions (Knust and Bossinger, 2002; Lecuit and Wieschaus, 2002; Nelson, 2003), adhesive structures that facilitate epithelial continuity. Germband extension and neighbour exchange are disrupted upon inhibition of endocytosis, the process by which cells internalize molecules (Levayer et al., 2011; Jewett et al., 2017). The core adherens junction protein E-cadherin is enriched in DV interfaces in the germband (Blankenship et al., 2006), and this pattern is disrupted when the endocytic machinery, which is preferentially active at AP edges (Levayer et al., 2011), is inhibited (Levayer and Lecuit, 2013). Together, these data suggest that polarized endocytic activity associated with actomyosin contractility drives junctional disassembly during germband extension. Interestingly, myosin is required for vesicle scission (Jewett et al., 2017), and polarized endocytosis orients medial myosin flows (Levayer and Lecuit, 2013), reflective of an intricate co-dependent relationship between cytoskeletal dynamics and junctional remodelling during cell intercalation.

The mechanisms that promote extension during cell intercalation are not well understood. Multicellular vertices resolve through the assembly of new DV interfaces. Actin is the first molecule known to localize to nascent DV interfaces, presumably to scaffold the new junction. A few minutes later, Bazooka localizes to DV interfaces (Blankenship et al., 2006), suggesting that Bazooka acts to stabilize newly forming DV interfaces as they form. Although myosin is excluded from the new interfaces, myosin phosphorylation mutants
display incorrectly oriented or unstable DV junctions (Kasza et al., 2014), suggesting that contractile forces are important to orient and maintain the elongation of interfaces during axis elongation. However, the cellular and molecular mechanisms that orient and promote vertex resolution are unclear.

Other cellular behaviours also contribute to tissue extension in the *Drosophila* germband. Cells elongate parallel to the AP axis as they intercalate (Figure 1.2D, top), and in mutants where cell intercalation is defective, cell deformation increases to partially compensate for it (Butler et al., 2009). Thus, intercalation and cell shape changes are independently controlled. It should be noted, however, that in mutants in which AP patterning is disrupted, cells elongate more than in the wild type, even though the germband only partially extends (Farrell et al., 2017). This indicates that while cell shape changes contribute to axis elongation, cell shape changes are not sufficient to complete extension of the body axis.

Oriented cell divisions also contribute to *Drosophila* axis elongation (Figure 1.2D, bottom). A population of cells at the posterior tip of the germband undergoes divisions oriented along the axis of extension (da Silva and Vincent, 2007). Consistent with a role for oriented cell division in axis elongation, disrupting AP patterning removes the AP bias of division orientation, and additionally, inhibition of cell division reduces the extent of germband extension (da Silva and Vincent, 2007). In addition, the mesectoderm, a group of cells that forms the ventral midline of the embryo and gives rise to a population of neurons and glia, also divide preferentially along the AP axis to alleviate the strain caused by cell intercalation in the adjacent ectoderm (Wang et al., 2017). Neither population participates in cell intercalation (da Silva and Vincent, 2007; Wang et al., 2017), representing an independent mechanism that shapes the *Drosophila* germband.

### 1.3 Germband extension involves tissue-tissue interactions

It has been suggested that external tissue-wide forces contribute to germband extension, promoting neighbour exchange and rosette resolution as a consequence. As indicated above, cells elongate parallel to the AP axis to compensate for disruptions in cell intercalation
(Butler et al., 2009), suggesting the presence of an external force that pulls cells along the AP axis. Germband extension is but one morphogenetic process in the *Drosophila* embryo that occurs during gastrulation, and the development of adjacent tissues, such as the internalization of the mesoderm and the posterior midgut, has the potential to affect ectodermal tissue movements. Recent studies have explored the extent by which axis elongation is coupled to other concomitant processes during *Drosophila* gastrulation.

Mesodermal precursors in *Drosophila* are internalized just prior to germband extension (Figure 1.3A). Mesoderm precursors at the ventral-most surface of the embryo coordinate the constriction of their apical surfaces to bend the tissue, which results in the internalization of the mesoderm (Martin et al., 2009). Simultaneously, the two rows of mesectoderm cells on either side of the mesoderm meet and form new contacts along the ventral midline, sealing the mesoderm inside the embryo. It was proposed that external forces supplied by mesoderm invagination acts to trigger the onset of germband extension, and that germband cells stretch along the DV axis as a result (Butler et al., 2009). However, the germband fully extends in mutants where full mesoderm specification is removed, and cell intercalation proceeds normally (Irvine and Wieschaus, 1994; Fernandez-Gonzalez et al., 2009), indicating that mesoderm invagination is unlikely to act as an external force that drives axis elongation.

The onset of germband extension coincides with the internalization of the midgut on the posterior end of the embryo (Leptin et al., 1992) (Figure 1.3B). Laser ablation studies revealed the presence of AP-oriented tension predominantly in the posterior region of the germband (Collinet et al., 2015; Lye et al., 2015), suggesting that a pulling force from the posterior end of the embryo might stretch the germband along its AP axis. In embryos where posterior midgut invagination is inhibited, germband extension is disrupted without affecting cell intercalation (Collinet et al., 2015) nor cell shape changes (Lye et al., 2015). Using a high-powered laser to cauterize or “glue” the germband to the protective sac that encloses the embryo further indicated the presence of an AP pulling force: in embryos in which mesoderm invagination was inhibited, cells posterior to the cauterization site still elongated along the AP axis, and tore away from the cauterized area, whereas these behaviours were abolished in embryos in which posterior midgut invagination was disrupted (Collinet et al.,
Figure 1.3: Morphogenetic processes adjacent to Drosophila germband extension.

(A) Mesoderm invagination. Lateral view of embryo (left) with transverse slice representing en face view (right). The mesoderm (yellow) folds into the embryo, pulling on the ectoderm (blue) as it invaginates; the mesectoderm (red) seals over the mesoderm on the surface of the embryo. (B) Invagination of the posterior midgut. During germband extension, the posterior midgut is internalized (green) thus pulling on the posterior tip of the germband (blue). Anterior left, dorsal up. (A-B) Time is in hours:minutes post-fertilization.
The contribution of forces from posterior midgut invagination is supported by *in silico* studies that suggest that an external AP pulling force could be contributing additional forces that orient and facilitate assembly of new interfaces during cell intercalation (Collinet et al., 2015; Siang et al., 2018). However, it should be noted that in posterior midgut invagination mutants the germband still extends, albeit to a lesser degree (Collinet et al., 2015; Lye et al., 2015), suggesting that posterior midgut invagination does not act alone in directing tissue extension. The mechanisms by which germband cells promote tissue extension autonomously remain unclear.

The cell behaviours driving germband extension (discussed later in this chapter) are conserved across species, and are also implicated in other convergence and extension processes, including primitive streak formation in chick embryos (Voiculescu et al., 2007); gut organogenesis (Chalmers and Slack, 2000), neural tube closure (Davidson and Keller, 1999), and elongation of kidney tubules (Lienkamp et al., 2012) in *Xenopus*; epiboly in *Xenopus* (Keller, 1980) and zebrafish (Warga and Kimmel, 1990); convergence and extension of the mesoderm in *Xenopus* (Wilson et al., 1989; Shih and Keller, 1992), zebrafish (Yin et al., 2008), and mouse (Yen et al., 2009); and visceral endoderm migration (Migeotte et al., 2010; Trichas et al., 2012), eye lid closure (Heller et al., 2014), neural plate elongation (Williams et al., 2014), palate fusion (Kim et al., 2015), and limb bud elongation (Lau et al., 2015) in mice. Therefore, characterizing the cell behaviours and molecular dynamics that drive axis elongation in *Drosophila* will inform our understanding of how cells communicate with each other to act cohesively during tissue morphogenesis, and will shed light on the origins of related developmental disorders. Furthermore, the identification of the signalling networks that govern convergence and extension will provide insight towards the development of regenerative medicine techniques and improved bioengineered tissues to treat developmental disorders, including neural tube defects such as spina bifida and anencephaly.
1.4 The ectoderm-mesectoderm interface during germband extension

Mesectoderm cells separate the internalized mesoderm from the lateral ectoderm, forming the ventral midline of the animal after the mesoderm is internalized. Mesectoderm cells are programmed prior to gastrulation with a cell identity distinct from that of the ectoderm and the mesoderm—while mesoderm cells express the transcription factor twist and the transcriptional repressor snail, mesectoderm cells express only twist, andectoderm cells express neither (Kosman et al., 1991; Rao et al., 1991). The transcription factor single-minded (sim) is a target of Twist that establishes mesectodermal identity (Kosman et al., 1991). In mesoderm cells, Snail represses sim, but misexpression of Sim in non-mesectoderm cells reprograms cell fate to mesectodermal (Nambu et al., 1991). In animals depleted of sim, the mesectodermal identity is deleted, and the number and organization of neurons in the lateral region of the late embryo is disrupted (Menne and Klambt, 1994). In the early embryo, mesectoderm cells remain adjacent to the ventral midline until they are internalized at approximately 9-10 hours post-fertilization. The cellular mechanisms that control internalization of the mesectoderm are not known.

Ectoderm and mesectoderm play different roles as the embryo develops. The ectoderm gives rise to the epidermis and the bulk of the central nervous system (Hartenstein and Campos-Ortega 1985), while the mesectoderm forms glia and a neuronal subset of the central nervous system upon its internalization (Jacobs and Goodman, 1989; Klambt et al., 1991; Wheeler et al., 2006). However, both the ectoderm and the mesectoderm originate from the same epithelium in the early embryo. Differences between these two tissues emerge as germband extension progresses—ectoderm cells intercalate (Irvine and Wieschaus, 1994; Bertet et al., 2004; Blankenship et al., 2006; Fernandez-Gonzalez et al., 2009), while mesectoderm cells do not (Butler et al., 2009; Lye et al., 2015; Wang et al., 2017). As axis elongation proceeds, mesectoderm cells will undergo one round of divisions oriented along the AP axis of the embryo, and subsequently the ectoderm completes three rounds of mitosis along random axes (Foe, 1989; Wang et al., 2017). In spite of the vigorous morphogenetic activity, ectoderm and mesectoderm remain spatially segregated through mechanisms that are currently unclear.
Mesectoderm and mesoderm precursors are also physically adjacent in the primitive epithelium that forms the *Drosophila* embryo. After their internalization, mesoderm cells rearrange and divide to form a monolayer inside the body cavity (Hartenstein and Campos-Ortega 1985). Mesectoderm and mesoderm cells remain connected throughout the process, and thus it is possible that the mesoderm pulls on the mesectoderm as it spreads. However, whether mesoderm-mesectoderm interactions play a role in the internalization of the mesectoderm is currently unknown.

### 1.5 Mechanisms of tissue boundary formation and maintenance

Highly controlled spatial and temporal patterning is critical for the progression of a stereotyped developmental program. It is strikingly remarkable that all cells within an organism originate from the same single-cell embryo, yet as morphogenesis proceeds, cell proliferation and differentiation readily intensifies to produce a number of diverse and specialized organs. During this process, cells of similar fate tend to be sequestered away from “unlike” cells in compartments, which are defined as groups of positionally confined cells exclusively from the same lineage (Crick and Lawrence, 1975). Isolating compartments from neighbouring groups can be achieved through the assembly of compartment boundaries, which can restrict cell-cell communication within subpopulations and prevent cell mixing during tissue morphogenesis. In addition to constraining fate-specific signals, boundaries can also direct differentiation (Dahmann et al., 2011), and upon temporally controlled breakdown, can enable tissue fusion, such as during the construction of the dual-atria, dual-ventricle heart in humans (Wessels et al., 1996). Thus, compartment boundaries are essential for tissue specification during development.

A number of human disorders arise from defective cell sorting. For instance, boundaries spatially confine cell proliferation within segments of the intestine in the *Drosophila* midgut (Tian et al., 2016). The ability of boundaries to restrict proliferation from spreading beyond predefined limits has been of particular interest in the context of tumour suppression. In the mouse intestine, boundaries can segregate cancerous cells from native tissues, and metastasis occurs only when the boundary is disrupted (Cortina et al., 2007) (Figure 1.4A). Likewise, in
Figure 1.4: Compartment boundaries isolate epithelial regions to direct tissue-tissue interactions.

(A) In the mouse intestine, tumour cells (purple) are compartmentalized away from epithelial tissue when boundary signalling is intact (left), but spread throughout the intestinal tissue when boundary signalling is impaired (right). (B) Rhombomeric boundaries in the zebrafish hindbrain at the 10-somite (14 hours post fertilization) stage of embryonic development. (C) Parasegmental boundaries in the early Drosophila epidermis. Boundaries are defined by an anterior Wingless stripe (cyan) and a posterior Engrailed stripe (red). (D) Compartments in the Drosophila wing disc. The AP boundary (dark orange) separates anterior (light orange) and posterior (yellow) compartments; the DV boundary (dark green) separates dorsal (dotted) and ventral (striped) compartments. (C-D) Anterior left, dorsal up.
the prostate gland, the boundary separating epithelial and stromal compartments is often lost as tumours become invasive (Foty and Steinberg, 2004). Compartment boundaries can also establish signaling hubs that prevent congenital disorder. Boundaries separate the seven rhombomeres of the vertebrate hindbrain, which enable specialized differentiation into distinct brain regions in the adult (Lumsden and Krumlauf, 1996; Moens et al., 1998) (Figure 1.4B). The same boundaries relay patterning cues to nearby neural crest cells to allow specification and innervation of discrete skeletal structures, and the absence of rhombomere boundaries can lead to craniofrontonasal syndrome (Twigg et al., 2004; Davy et al., 2006). Thus, understanding how tissue boundaries are formed and maintained in vivo can uncover mechanisms by which impaired tissue boundaries contribute to the onset and spread of disease.

Characterizing tissue boundaries in vivo may prove useful to build artificial tissues (McGuigan and Javaherian, 2016). Many novel tissue engineering strategies are being developed that mimic in vivo development. However, boundary conditions have proven difficult to replicate in vitro, and thus tissue architecture is difficult to maintain in artificial tissues (Javaherian et al., 2013). Hence, studying the mechanisms of boundary formation and maintenance will facilitate the development of strategies for the successful generation of biomimetic artificial tissues.

1.5.1 Differences in gene expression establish compartment boundaries
Many efforts have been invested in identifying the molecular mechanisms that regulate tissue boundaries. Perhaps the most straightforward hypothesis proposed to organize tissue compartmentalization is that cells are genetically programmed to segregate into compartments from early stages of development.

Early on in Drosophila embryogenesis, the trunk of the body is patterned by maternal polarity genes that set up striped gene expression parallel to the DV axis (Sanson, 2001). Two of these genes, the transcription factor Engrailed and the secreted protein Wingless, form adjacent stripes, and at the interface between the two stripes, parasegmental boundaries
form that specify the different thoracic segments (Sanson et al., 1999; Tetley et al., 2016) (Figure 1.4C). During this process, segmental borders exhibit increasing linearity, preventing cell mixing (Monier et al., 2010) in a process essential for embryonic viability (Baker, 1988). Other boundaries are also dependent on similar polarity cues. For example, the boundary separating anterior and posterior cell populations in the epithelial sac that originates the wings (the wing imaginal disc) arises from the border between embryonic parasegments 4 and 5—the wing disc inherits a stripe of Engrailed which is required to orient boundary assembly (Morata and Lawrence, 1975; Blair and Ralston, 1997; Dahmann and Basler, 2000).

Boundary formation does not necessarily rely exclusively on embryonic patterning cues. Well into larval development, the imaginal wing disc forms a second boundary separating dorsal and ventral cells, giving rise to four compartments (Garcia-Bellido et al., 1976) (Figure 1.4D). Notch signalling at the dorsal-ventral boundary emerges first at a stripe of cells wherein the DV boundary will form (Michelli and Blair, 1999; Milan and Cohen, 2003). Ectopic expression of Notch in the wing disc is sufficient to generate boundary-like structures exhibiting increased linearity, even in instances where cells on either side of the “boundary” are both dorsal or ventral in origin (Rauskolb et al., 1999; O'Keefe and Thomas, 2001; Major and Irvine, 2005). Conversely, suppressing Notch activity results in DV boundary disruption (Major and Irvine, 2006). Thus, specific patterns of gene expression contribute to the formation of compartment boundaries. But how are these compartment boundaries assembled at the cellular and molecular levels?

1.5.2 Cell sorting through differential adhesion and cell repulsion

A hallmark of compartment boundaries is their characteristic linearity, a typical feature of cell sorting. One mechanism that has been long proposed to mediate cell sorting is the Differential Adhesion Hypothesis (Steinberg, 1963). The idea is that cells of unlike origin are able to recognize and avoid each other due to differences in adhesive properties, such as the expression of dissimilar adhesion receptors between the two cell types. For example, in the wing disc, the expression of the junctional cadherin Cad99 is elevated in a group of cells
directly anterior to the anterior boundary (Schlichting et al., 2005), suggesting that cells expressing Cad99 may have increased affinity for each other than for cells that do not express that cadherin. Adhesion has also been proposed to sort neighbouring neuroepithelial populations in the mouse brain, where the junctional proteins R-cadherin and cadherin-6 are complementarily expressed between two telencephalic compartments (Inoue et al., 2001). Importantly, in the *Drosophila* egg chamber, the oocyte selectively attaches itself to follicle cells expressing higher levels of E-cadherin, and cannot maintain its position in the posterior chamber when E-cadherin is downregulated (Godt and Tepass, 1998; Gonzalez-Reyes and St Johnston, 1998). Junctional adhesion is therefore a key regulator of tissue compartmentalization.

The Eph/ephrin receptor/ligand pair has also been implicated in boundary formation. Eph/ephrin establish compartment boundaries in the hindbrain (Xu et al., 1995; Chan et al., 2001; Cooke et al., 2001) and eye field (Xu et al., 1996; Cooke et al., 1997; Cavodeassi et al., 2013) in zebrafish, as well as the mesoderm-ectoderm interface (Rohani et al., 2011; Rohani et al., 2014) and the notochord–presomitic mesoderm border (Fagotto et al., 2013; Rohani et al., 2014) in *Xenopus*. In vertebrates, Eph receptors and ephrin ligands exist in multiple isoforms and do not necessarily bind to each other with specificity (Gale et al., 1996; Himanen et al., 2004). They are also capable of bidirectional signalling—that is, receptor-ligand binding can trigger downstream effects originating from Eph, which can be completely different from the effects originating from ephrin (Pasquale, 2008; Klein, 2012). The ability of the Eph/ephrin family to exhibit differences in expression levels and membrane composition across cell types, as well as its capacity for promiscuous binding, have been proposed to be key characteristics enabling compartmentalization. Importantly, Eph/ephrin signaling can control boundary formation by up- or downregulating cell-cell adhesion. For example, ephrinB1 can outcompete the small GTPase Cdc42 to bind to Par-6, disrupting tight junction assembly in the *Xenopus* ectoderm (Lee et al., 2008); and Eph/ephrin signalling cannot maintain compartmentalization of tumour cells in the mouse intestinal epithelium when E-cadherin expression was silenced (Cortina et al., 2007). During mesoderm-ectoderm fusion in *Xenopus* embryos, each germ layer expresses a unique set of Eph receptors and ephrin ligands, in turn mediating cell segregation (Rohani et al., 2011).
Live imaging revealed that the interface between mesoderm and ectoderm underwent cycles of adhesion and detachment, which the authors termed cell repulsion (Rohani et al., 2011). Further study revealed that cyclical repulsion relies on a fine balance between Eph/ephrin signalling and junctional cadherin; detachment became more frequent when cadherins were depleted, while attachment increased when cadherins were overexpressed (Rohani et al., 2014). The balance of Eph/ephrin across cell-cell contacts controlled the degree of cell repulsion, as ectopically expressing ectodermal Eph/ephrin pairs in the mesoderm induces increased detachment rates with the surrounding mesoderm unaffected (Rohani et al., 2014). Thus, Eph/ephrin mediate boundary formation by regulating levels of cell-cell adhesion between unlike populations.

Differences in cell adhesion may not be necessary for boundary formation. Adhesion proteins that are differentially expressed across the compartment boundaries in the Drosophila wing disc have been identified, and segregation is disrupted when they are overexpressed, but peculiarly, it is unaffected in loss of function experiments (Milan et al., 2001; Schlichting et al., 2005). Even more mysteriously, two independent screens were conducted to find boundary regulators at wing disc boundaries, and neither produced hits that were known to control cell adhesion properties (Vegh and Basler, 2003; Bejarano et al., 2008). This suggests that although adhesion can play a role in cell-cell compartmentalization, it could be acting secondarily or redundantly to other mechanisms of boundary organization.

1.5.3 Cytoskeletal contractility can resist cell mixing

Further evidence later challenged the idea that differential adhesion acts as the main mechanism directing compartmentalization. For example, the junctional proteins E-cadherin, α-catenin, and β-catenin are not enriched at the dorsal-ventral boundary of the Drosophila wing disc any more so than at junctions within compartments (Major and Irvine, 2005, 2006), raising the possibility that mechanisms other than adhesion may be acting to segregate cell groups. Instead, the focus began to shift towards determining how boundary morphology, and in particular its straight appearance, could be regulated. Notably, both actin and myosin are elevated specifically between compartments in the wing disc, and mutants for
capulet (a regulator of actin polymerization) and zipper (the myosin heavy chain subunit) abolished the hallmark linearity of the boundary (Major and Irvine, 2005, 2006). These data suggest that compartmentalization could be mandated by cytoskeletal contractility.

Many studies have since identified actomyosin enrichment at boundaries, including at the anterior-posterior compartment boundary in the wing disc (Landsberg et al., 2009; Umetsu et al., 2014a), the epidermal parasegmental boundaries (Monier et al., 2010; Tetley et al., 2016; Scarpa et al., 2018; Urbano et al., 2018), and around precursor salivary gland placode cells (Sanchez-Corrales et al., 2018) in Drosophila; at the mesoderm-ectoderm boundary (Rohani et al., 2011) and between the notochord and presomitic mesoderm (Fagotto et al., 2013) in Xenopus; between germ layers (Krieg et al., 2008), around the eye field (Cavodeassi et al., 2013), and at the borders delimiting hindbrain segments (Calzolari et al., 2014) in zebrafish; and within the neuroepithelium in mice (O'Neill et al., 2016). Laser ablation experiments have confirmed that tissue boundaries are under increased tensile stress, both in vivo (Landsberg et al., 2009; Aliee et al., 2012; Tetley et al., 2016; Urbano et al., 2018) and in vitro (Javaherian et al., 2015), suggesting that upregulated cytoskeletal contractility specifically at boundaries is responsible for its characteristic linearity. Indeed, reduced boundary straightness was observed through pharmacological inhibition of actomyosin contractility (Landsberg et al., 2009; Calzolari et al., 2014; O'Neill et al., 2016) and by locally disrupting myosin activity using fluorophore-assisted laser inhibition (FALI) (Monier et al., 2010). Thus, actomyosin-based contractility plays an important role in boundary maintenance.

How actomyosin is maintained at tissue boundaries remains uncertain. In other systems, myosin dynamics can be regulated by tension. Myosin has higher affinity for actin when it is stabilized in its ADP-bound form under tensile stress in vitro (Kovacs et al 2007), and micropipette aspiration can recruit myosin to the site of increased tension in Dictyostelium (Effler et al 2006). Similar aspiration studies, in conjunction with laser ablation and turnover experiments, identified a positive feedback mechanism between myosin and tension in the contracting epidermal cables in the Drosophila germband (Fernandez-Gonzalez et al 2009). Furthermore, at the embryonic wound margin in Drosophila, myosin concentration at the
wound edge accumulates as the wound closes (Fernandez-Gonzalez and Zallen 2013). Myosin recruitment to the wound edge is deformation-dependent, and tension increases as wound repair progresses, which was found to play a key role in stabilizing myosin at the wound margin (Kobb et al 2017; Zulueta-Coarasa and Fernandez-Gonzalez 2018). However, the relationship between tension and myosin dynamics at tissue boundaries remains unexplored.

1.6 Do mesectoderm fate and function depend on its position?

It has been suggested that the positioning of the mesectoderm at the ventral midline plays a large role in its functions during late embryogenesis. In embryos in which sim is expressed in a group of ectoderm cells to change their fate to mesectoderm, the mesectoderm still forms at the interface between ectoderm and mesoderm; however, the mesectoderm cells induced on the lateral surface of the embryo now replace epidermal cells in the ectoderm, disrupting epithelial function (Nambu et al., 1991). Thus, it is unclear whether mesectoderm differentiation and function depends on proper cell positioning at the ventral surface of the embryo, correct specification of the mesectoderm fate, or both.

Mesectoderm position on the ventral surface is challenged during embryonic development. The ectoderm intercalates during germband extension (Irvine and Wieschaus, 1994; Bertet et al., 2004; Blankenship et al., 2006; Fernandez-Gonzalez et al., 2009), and both ectoderm and mesectoderm undergo cell divisions (Foe, 1989; da Silva and Vincent, 2007; Wang et al., 2017). Cell intercalation and cell division could both promote tissue intermixing. However, compartment boundaries formed by actomyosin cables under tension can maintain tissue boundaries even in the presence of cell intercalation or division (Monier et al., 2010; Umetsu et al., 2014a; Tetley et al., 2016; Scarpa et al., 2018). Thus, the mesectoderm-ectoderm interface could be formed by a cytoskeletal cable that generates mechanical forces to segregate the two populations, a possibility that has not been investigated.
1.7 Thesis overview and objectives

The *Drosophila* germband provides an excellent system to study the mechanisms of cell intercalation and tissue boundary formation, two behaviours that recur throughout development and are conserved across species. The work presented herein will investigate the mechanisms of cell intercalation and tissue boundary formation during *Drosophila* axis elongation. To this end, I have completed the following aims:

1. Establish the role of local mechanical forces in driving junctional assembly during cell intercalation.
   a. I used confocal time-lapse microscopy and pharmacological manipulations to determine the role of anisotropic cell oscillations in the formation of new junctions.
   b. I developed techniques utilizing laser ablation to alter ectopic tension and study its effect on nascent interfaces.
   c. I found that forces originating from neighbouring cells are both necessary and sufficient for the assembly of nascent interfaces during tissue extension.

2. Characterize the compartment boundary between mesectoderm and ectoderm cells that forms during axis elongation.
   a. I identified molecular asymmetries that are necessary for boundary formation, including the presence of supracellular myosin cables at the interfaces between ectoderm and mesectoderm.
   b. I used laser ablation and Fluorescence Recovery After Photobleaching to establish that tension regulates myosin accumulation and turnover at the ectoderm-mesectoderm boundary.
   c. Using biophysical and pharmacological manipulations, I showed that myosin cables act as ropes that maintain the mesectoderm on the surface of the embryo.

The results presented in this thesis highlight the importance of biomechanical signalling during embryonic development. In Chapter 2, I examine how anisotropic tensile forces exerted on multicellular vertices can facilitate directional junctional assembly. In Chapter 3, I show that supracellular forces can maintain tissue boundaries to segregate ectoderm from
mesectoderm. Together, these data demonstrate how mechanical signals generated from cell-cell interactions can translate into tissue-level dynamics, driving tissue extension or maintaining tissue-tissue compartmentalization during embryonic development.
Chapter 2: Local mechanical forces promote polarized junctional assembly and axis elongation in *Drosophila*

A modified version of this chapter appeared as:


**Contributions:** JCY and RFG conceived of the study, designed experiments, and wrote the manuscript. JCY performed all experiments and data analysis in this chapter.
Chapter 2: Local mechanical forces promote polarized junctional assembly and axis elongation in *Drosophila*

2.1 Abstract

Axis elongation is a conserved process in which the head-to-tail or anterior-posterior (AP) axis of an embryo extends. In *Drosophila*, cellular rearrangements drive axis elongation. Cells exchange neighbours by converging into transient multicellular vertices which resolve through the assembly of new cell interfaces parallel to the AP axis. We found that new interfaces elongate in pulses correlated with periodic contractions of the surrounding cells. Inhibiting actomyosin contractility globally, or specifically in the cells around multicellular vertices, disrupted the rate and directionality of new interface assembly. Laser ablation indicated that new interfaces sustained greater tension than non-elongating ones. We developed a method to apply ectopic tension and found that increasing AP tension locally increased the elongation rate of new edges by more than 2-fold. Increasing dorsal-ventral tension resulted in vertex resolution perpendicular to the AP direction. We propose that local, periodic contractile forces polarize vertex resolution to drive *Drosophila* axis elongation.

2.2 Introduction

Axis elongation is a conserved morphogenetic process in which the basic body plan of an animal is established. In vertebrates, axis elongation involves convergence and extension movements mediated by cell intercalation, cell migration, and oriented cell division (Benazeraf and Pourquie, 2013). In *Drosophila*, axis elongation occurs in an epithelial monolayer referred to as the germband, which lengthens by more than two-fold along the anterior-posterior (AP) axis of the animal, while narrowing along the dorsal-ventral (DV) axis (*Chapter 1*, Figure 1.1B). The changes in germband architecture are largely driven by cell intercalation (Irvine and Wieschaus, 1994).
Cell intercalation facilitates changes in tissue architecture through neighbour exchange events. In vertebrates, cell intercalation drives many developmental processes, including primitive streak formation in chick embryos (Voiculescu et al., 2007); gut organogenesis (Chalmers and Slack, 2000), neural tube closure (Davidson and Keller, 1999), and elongation of kidney tubules (Lienkamp et al., 2012) in *Xenopus*; epiboly in *Xenopus* (Keller, 1980) and zebrafish (Warga and Kimmel, 1990); convergence and extension of the mesoderm in *Xenopus* (Wilson et al., 1989; Shih and Keller, 1992), zebrafish (Yin et al., 2008), and mouse (Yen et al., 2009); and visceral endoderm migration (Migeotte et al., 2010; Trichas et al., 2012), eye lid closure (Heller et al., 2014), neural plate elongation (Williams et al., 2014), palate fusion (Kim et al., 2015), and limb bud elongation (Lau et al., 2015) in the mouse.

During *Drosophila* axis elongation, cell intercalation is driven by polarized actomyosin contractility, which promotes the disassembly of interfaces separating anterior and posterior cell neighbours (AP interfaces), to form multicellular vertices where four or more cells converge (Bertet et al., 2004; Zallen and Wieschaus, 2004; Blankenship et al., 2006). Polarized disassembly of cell contacts is also associated with cell intercalation in chick (Rozbicki et al., 2015), *Xenopus* (Shindo and Wallingford, 2014), and mouse embryos (Williams et al., 2014; Lau et al., 2015). Following contraction of AP interfaces in the *Drosophila* germband, multicellular vertices are systematically resolved through the assembly of new contacts separating dorsal and ventral cell neighbours (DV interfaces, Figure 2.1). While vertex resolution and the subsequent assembly of new cell-cell interfaces drive tissue elongation, little is known about the mechanisms that regulate these processes. Myosin turnover between phosphorylated and unphosphorylated states is important for the directionality of vertex resolution (Kasza et al., 2014). Computational modelling suggests that periodic contraction of the apical surface of germband cells, driven by pulsatile actomyosin networks, could promote the oriented assembly of new cell contacts (Lan et al., 2015). However, the role of actomyosin contractility in vertex resolution remains unclear.

In this study, we combine quantitative imaging with biophysical and pharmacological manipulations to investigate the mechanisms of vertex resolution in *Drosophila* axis elongation. We find that the assembly of new interfaces during vertex resolution occurs in
Figure 2.1: Axis elongation in *Drosophila* is driven by neighbour exchange events.

Diagram (top) and germband cells (bottom) showing a neighbour exchange event. An AP interface contracts (left, green), forming a vertex where four cells meet (centre, magenta). The vertex resolves through the assembly of a new DV interface (right, cyan). Scale bar, 5 µm. Anterior left, dorsal up.
pulses associated with the periodic contraction of the cells anterior and posterior to the multicellular vertex. Pulsed actomyosin contractility in the cells around the vertex is critical for the directionality and rate of assembly of the new cell interface. Local, ectopic AP tension is sufficient to accelerate the assembly of new interfaces, and local DV tension can reorient vertex resolution. Together, our results demonstrate that local, periodic actomyosin contractility directs the resolution of multicellular vertices and promotes the assembly of new cell contacts during polarized cell rearrangements in *Drosophila* germband extension.

### 2.3 Materials and Methods

#### 2.3.1 Fly stocks

We used the following markers for live imaging: *ubi-E-cadherin:GFP* (Oda and Tsukita, 2001), *sqh-sqh:mCherry* (Martin et al., 2009), *resille:GFP* (Morin et al., 2001), *sqh-GFP:utrophin* (Rauzi et al., 2010), and *par-6Δ226, par-6:GFP* (Wirtz-Peitz et al., 2008).

#### 2.3.2 Time-lapse imaging

Stage 7 embryos were dechorionated in 50% bleach for 90 s, rinsed, glued ventrolateral side down to a glass coverslip using heptane glue, and mounted in a 1:1 mix of halocarbon oil 27 and 700 (Sigma-Aldrich, St. Louis, MO). Embryos were imaged using a Revolution XD spinning disk confocal microscope equipped with an iXon Ultra 897 camera (Andor, Belfast, UK) and a 1.5x coupling lens. For experiments using laser ablation, a 60x oil immersion lens (Olympus, Shinjuku, Japan; NA 1.35) was used; for all other experiments, a 40x oil immersion lens (Olympus, NA 1.35) was used. Sixteen-bit Z-stacks were acquired at 0.3 μm steps every 3-10 s (8-10 slices per stack).

#### 2.3.3 Laser ablation

Ablations were induced using a pulsed Micropoint N2 laser (Andor) tuned to 365 nm. The laser delivers 120 μJ pulses at durations of 2-6 ns each. For ablation of cell boundaries, 10 consecutive laser pulses were delivered to a single spot along a cell interface. For single-cell wounds, 10 consecutive laser pulses were delivered to each of two spots spaced 2 μm apart.
on the medial-apical region of the cell of interest. In experiments where local tension was reduced, 10 laser pulses were delivered to a single spot on the medial-apical region of the cell of interest. Cells were re-ablated upon assembly of medial-apical myosin networks. In sham-irradiated controls, cells were targeted with the laser completely attenuated every 60 seconds to mimic the repeated ablations performed in the corresponding experiments.

2.3.4 Drug injections

Embryos were dechorionated and glued to a coverslip as above, dehydrated for 10-15 minutes, and covered with a 1:1 mix of halocarbon oil 27 and 700 (Sigma-Aldrich). Embryos were injected using a Transferman NK2 micromanipulator (Eppendorf, Hamburg, Germany), and a PV820 microinjector (WPI, Sarasota, FL) attached to the spinning disk confocal microscope. Drugs (Y-27632, Tocris Bioscience, Bristol, UK) and (Cytochalasin D, EMD Millipore, Darmstadt, Germany) were injected into the perivitelline space, where they are predicted to be diluted 50-fold (Foe and Alberts, 1983). Y-27632 was injected at 100 mM in water; control embryos were injected with water. Cytochalasin D was injected at 5 mM in 50% DMSO; control embryos were injected with 50% DMSO. Embryos were imaged immediately after injection for at least 10 minutes.

2.3.5 Cell segmentation, tracking, and quantification

Image analysis was performed using algorithms developed with Matlab (MathWorks, Natick, MA) and DIPImage (Delft University of Technology, Delft, Netherlands) and integrated in our custom Scientific Image Segmentation and Analysis (SIESTA) software (Fernandez-Gonzalez and Zallen, 2011; Leung and Fernandez-Gonzalez, 2015).

The onset of vertex resolution was established as the first time at which the length of a nascent interface exceeded 1 µm. New edge orientation was quantified relative to the AP axis of the embryo, defined as 0°, and was measured 150 s after the onset of vertex resolution. Edge length was measured as the distance between the two vertices defining the edge. To measure how fast new edges assemble, we defined the rate of elongation at time $t$ as:
rate of elongation \( (t) = \frac{l(t) - l(t_0)}{t - t_0}, \)  \hspace{1cm} (2.1)

where \( l(t) \) represents the length of the edge at time \( t \), and \( t_0 \) is the time of onset of vertex resolution. The rate of elongation was calculated over the initial 90 s of interface elongation, unless indicated otherwise. Cell areas were quantified using an algorithm in which seeds were manually placed within each cell of interest in the first timepoint of a movie. Seeds were automatically expanded to delineate the cell boundaries using the watershed method (Beucher, 1992), a region-growing algorithm. Seeds were subsequently propagated to the next time point using particle image velocimetry to account for cellular movement (Wang and Fernandez-Gonzalez, in preparation), and the process was iterated. To measure retraction velocities following laser ablation, we determined the change in distance between the two vertices delimiting the ablated interface, and divided this value by the sum of the ablation and the stack acquisition times.

In time-lapse images, fluorescence was measured from maximum intensity projections of three apical slices. Fluorescence intensities were background-subtracted using the most frequent pixel value (the mode) of a maximum intensity projection of three basal slices cropped around the region of interest (10 µm x 10 µm). Intensity values were corrected for photobleaching by dividing by the mean image intensity in each time point. To quantify myosin levels in new DV edges with respect to AP edges, we imaged embryos expressing myosin:mCherry, and measured fluorescence in manually traced cell interfaces. We subtracted the image mode from the myosin fluorescence measurements as an estimate of the background.

Oscillatory cell behaviours were characterized by the rate of change per minute of the corresponding magnitude, calculated as the difference of measurements collected one minute apart. To calculate periods, rates of change were detrended by subtracting the line of best fit using the \textit{detrend} function in Matlab (Mathworks). The period was computed as the inverse of the dominant frequency in a fast Fourier transform of the detrended signal. To calculate the mean change in edge length during the elongation or shortening steps of new DV edge formation, we quantified the area under the curve for positive (elongation) or negative
(shortening) rates of length change. The resulting numbers were the total elongation or shortening for a given edge, which divided by the number of pulses, yielded the mean change in length per elongation or shortening pulse. The correlation between signal pairs was determined using the *corrcoef* function in Matlab (Mathworks). To find the time shift required for minimum or maximum correlation between signal pairs, one signal was shifted forward and backward in time relative to the other, in increments of 10 s up to 240 s. With each increment, the correlation was recalculated. The resulting correlation curve was Gaussian-smoothed using a sigma of 10 s, and the time shifts required to obtain the first local minimum and maximum in the correlation values were determined.

### 2.3.6 Statistical analysis

Sample means were compared using Student’s *t*-test (Glantz, 2002). The significance of correlation coefficients was calculated by transforming the correlation value into a *t*-statistic using the Matlab *corrcoef* function (Mathworks). Sample distributions were contrasted using Kolmogorov-Smirnov’s test. Error bars indicate the standard error of the mean (s.e.m.).

### 2.4 Results

#### 2.4.1 Pulsed assembly of new junctions during germband extension

To investigate the mechanisms of vertex resolution during *Drosophila* axis elongation, we used quantitative image analysis to measure the dynamics of assembly of new DV junctions in embryos expressing Resille:GFP (Morin et al., 2001) to visualize cell outlines. We found that the assembly of new DV edges occurred in cycles of elongation and shortening (Figure 2.2A-B, blue line), with a period of 126±5 s (n = 110 edges). On average, elongation pulses increased edge length by 772±46 nm, while shortening pulses decreased edge length by a significantly smaller amount, 114±19 nm (n = 110 edges, *P* = 9.0x10^-22), thus resulting in net edge elongation. Germband cells undergo characteristic cycles of apical area contraction and relaxation with a period of 130±3 s, and predominantly oriented along the AP axis of the embryo (Fernandez-Gonzalez and Zallen, 2011; Sawyer et al., 2011). To examine whether the anisotropic oscillations of germband cells were associated with the assembly of new cell
junctions during vertex resolution, we compared the changes in length of the nascent DV edge to the changes in apical area of the cells immediately anterior or posterior to that DV edge (Figure 2.2A-B). In a majority of cases (143/220 cell-edge pairs, 65%) we observed a negative correlation between changes in length of the new DV junction and changes in area of the cell anterior or posterior to it (Figure 2.2C). To calculate the dominant relationship between changes in anterior/posterior cell area and new DV edge length, we quantified the correlations after shifting the edge length backward or forward in time. Reaching the maximum correlation with small time shifts would indicate in-phase oscillations, while maximum anti-correlation with small time shifts would suggest oscillations in anti-phase. We found that short time shifts of the edge length signal maximized the anti-correlation, while longer time shifts were necessary to maximize the correlation ($P = 1.74 \times 10^{-5}$, Figure 2.2D-E), further suggesting that pulses of new DV edge assembly are associated with the contraction of the anterior and posterior cells. Similar analyses demonstrated that changes in length of the new edge were predominantly positively correlated with changes in the apical area of the dorsal and ventral cells, which share the new edge (156/220 cell-edge pairs, 71%, Figure 2.3). Together, our results suggest that pulsed contractions of the cells in the immediate vicinity of a multicellular vertex may promote vertex resolution during *Drosophila* axis elongation.

2.4.2 Actomyosin-induced tension is necessary and sufficient for directional vertex resolution

The cyclical changes of apical area in germband cells are driven by pulsatile networks of medial-apical non-muscle myosin II (Rauzi et al., 2010; Fernandez-Gonzalez and Zallen, 2011; Sawyer et al., 2011). To investigate if actomyosin contractility is necessary for vertex resolution, we injected embryos expressing E-cadherin:GFP and myosin:mCherry with the Rho-kinase inhibitor Y-27632 at 100 mM. Rho-kinase is one of the main activators of myosin (Amano et al., 1996; Kimura et al., 1996), and treatment with Y-27632 abolishes the ability of germband cells to generate mechanical force (Fernandez-Gonzalez et al., 2009). In Y-27632-injected embryos, germband cells displayed a rapid loss of myosin from their apical surface (Figure 2.4A), resulting in a dramatic reduction in the amplitude of apical area.
Figure 2.2: Directional assembly of new interfaces during vertex resolution is associated with pulsatile apical contractions and requires contractile activity.

(A) Vertex resolution during axis elongation in an embryo expressing Resille:GFP. Blue indicates the new DV interface, red labels the anterior and posterior cells. (A') Kymograph illustrating the elongation of the DV interface shown in (A). Scale bar, 10 s. The interface is rotated by 90° with respect to (A). Anterior down, dorsal left. (B) Rates of change for edge length (blue, solid line), anterior cell area (red, dashed line), and posterior cell area (red, dotted line) during the neighbour exchange event shown in (A). Rate of change was calculated with respect to \( t + 60 \) s. (C) Correlation coefficients between changes in edge length and changes in anterior or posterior cell area (\( n = 220 \) pairs in 110 neighbour exchange events in 13 embryos). (D) Changes in correlation between edge length and anterior (dashed) or posterior (dotted) cell area during the neighbour exchange event shown in (A) when the edge length signal was shifted in time in 10-second increments. Arrowheads
indicate the correlation minima (blue) or maxima (red) closest to 0 s shift. (E) Distribution of time shifts (absolute value) required to obtain the minimum (blue) and maximum (red) correlations in all 220 signal pairs shown in (C). (F-G) Rate of change in cell area in embryos injected with water (F, \( n = 122 \) cells in 3 embryos) or 100 mM Y-27632 (G, \( n = 99 \) cells in 3 embryos). Each line represents a single cell. (H) Oscillation amplitude for changes in cell area in embryos injected with water (blue) or 100 mM Y-27632 (red). Asterisks indicate \( P < 0.001 \). (I-J) Vertex resolution during axis elongation in embryos expressing E-cadherin:GFP and injected with water (I) or with 100 mM Y-27632 (J-J'). Arrowheads indicate nascent DV interfaces. (K) Distribution of vertex resolution angles relative to the AP axis in embryos injected with water (blue, \( n = 28 \) vertices in 3 embryos) or 100 mM Y-27632 (red, \( n = 25 \) interfaces in 3 embryos). Angles were measured 150 s after the onset of vertex resolution. An angle of 90° with respect to the AP axis corresponds to the DV axis. (L) Length of new DV interfaces forming within 30° of the AP axis in embryos injected with water (blue, \( n = 25 \) interfaces in 3 embryos) or 100 mM Y-27632 (red, \( n = 11 \) interfaces in 3 embryos). (A, I-J') Anterior left, dorsal up. Scale bars, 5 \( \mu \)m. (B, F-G, L) Time is with respect to the onset of vertex resolution, defined as the first time point in which the length of the nascent interface exceeded 1 \( \mu \)m. (H, K-L) Error bars, s.e.m.
Figure 2.3: Dorsal and ventral cells oscillate with new DV interfaces.

(A) Vertex resolution during axis elongation in an embryo expressing Resille:GFP. Blue indicates the new DV interface, red labels the dorsal and ventral cells. Anterior left, dorsal up. Scale bar, 5 µm. (B) Rates of change for edge length (blue, solid line), dorsal cell area (red, dashed line), and ventral cell area (red, dotted line) during the neighbour exchange event shown in (A). Rate of change was calculated with respect to $t + 60$ s. (C) Correlation coefficients between changes in edge length and changes in dorsal or ventral cell area ($n = 220$ pairs in 110 neighbour exchange events in 13 embryos). (D) Changes in correlation between edge length and dorsal (dashed) or ventral (dotted) cell area during the neighbour exchange event shown in (A), when the edge length signal was shifted in time in 10-second increments. Arrowheads indicate the correlation minima (blue) or maxima (red) closest to 0 s shift. (E) Distribution of time shifts (absolute value) required to obtain the minimum (blue) and maximum (red) correlations in all 220 signal pairs shown in (C).
Figure 2.4: Directional assembly of new DV interfaces during vertex resolution requires actomyosin contractility.

(A-A’’) Germband cells expressing E-cadherin:GFP (green, A’) and myosin:mCherry (magenta, A”), before (pre-injection) and at different times after injection with 100 mM Y-27632. Anterior left, dorsal up. Scale bars, 5 µm. (B-C) Vertex resolution during axis elongation in embryos expressing Resille:GFP and injected with water (B) or with 100 mM Y-27632 (C-C’). Arrowheads indicate nascent DV interfaces. Anterior left, dorsal up. Scale bars, 5 µm. (D) Distribution of vertex resolution angles relative to the AP axis in embryos injected with water (blue, n = 26 vertices in 3 embryos) or 100 mM Y-27632 (red, n = 43 interfaces in 7 embryos). Angles were measured 150 s after the onset of vertex resolution. An angle of 90° with respect to the AP axis corresponds to the DV axis. Error bars, s.e.m. (E) Length of new DV interfaces forming within 30° of the AP axis in embryos injected with water (blue, n = 21 interfaces in 3 embryos) or 100 mM Y-27632 (red, n = 19 interfaces in 7 embryos). Time is with respect to the onset of vertex resolution, defined as the first time point in which the length of the nascent interface exceeded 1 µm. Error bars, s.e.m.
oscillation \((P = 1.7 \times 10^{-44}, \text{Figure 2.2F-H})\). Inhibiting actomyosin contractility affected the directionality of vertex resolution: 9/25 vertices resolved within 30° of the DV axis in Y-27632-injected embryos, in contrast to 0/28 in water-injected controls \((P = 0.02, \text{Figure 2.2I, J', K})\). In addition, for vertices that resolved along the AP axis, inhibiting Rho-kinase reduced the rate of new edge elongation with respect to controls \((0.001 \pm 0.080 \, \mu\text{m/min} \text{ vs.} 0.28 \pm 0.06 \, \mu\text{m/min}, \text{respectively, } P = 0.01, \text{Figure 2.2I-J, L})\), suggesting that myosin activity facilitates the assembly of new DV interfaces. Similar results were obtained in embryos expressing Resille:GFP, a different cell outline marker (Figure 2.4B-E). However, Rho-kinase activity can regulate the localization of the Par polarity complex (Atwood and Prehoda, 2009; Simoes et al., 2010) (Figure 2.5), raising the possibility that abnormal vertex resolution upon Y-27632 injection was a consequence of defects in cell polarity, rather than reduced actomyosin contractility.

To further investigate the role of mechanical forces in vertex resolution, we disrupted actomyosin contractility by injecting embryos with 5 mM of Cytochalasin D, a drug that blocks actin polymerization by binding to the elongating end of filaments and preventing the addition of new actin monomers (Flanagan and Lin, 1980). Cytochalasin D injection disrupted the actin cytoskeleton (Figure 2.6A-B) and reduced apical area oscillations \((P = 0.04, \text{Figure 2.6C-E})\), without affecting the localization of Par-6, a member of the Par complex (Figure 2.5B). Cytochalasin D treatment led to an 83% reduction in the rate of new DV edge assembly with respect to controls \((0.07 \pm 0.10 \, \mu\text{m/min} \text{ vs.} 0.40 \pm 0.05 \, \mu\text{m/min}, \text{respectively, } P = 0.01, \text{Figure 2.6F-G, J})\). 4/15 vertices resolved along the DV axis in Cytochalasin D-injected embryos, in contrast to 0/50 in DMSO-injected controls \((P = 4.0 \times 10^{-15}, \text{Figure 2.6F, H-I})\). Strikingly, in Cytochalasin D-injected embryos, 32/47 vertices persisted for at least 10 min and up to 40 min (Figure 2.6K). Together, our results demonstrate that actomyosin contractility is necessary for the directional assembly of new interfaces during vertex resolution in Drosophila axis elongation.
Figure 2.5: Par complex localization is affected by Y-27632, but not by Cytochalasin D.

(A-B) Germ band cells expressing Par-6:GFP at endogenous levels and injected with water (A), 100 mM Y-27632 in water (A’), 50% DMSO (B) or 5 mM Cytochalasin D in 50% DMSO (B’). Anterior left, dorsal up. Scale bars, 10 µm.
Figure 2.6: Oriented assembly of new DV interfaces requires actin-based contraction.

(A-B) Germband cells expressing GFP:utrophin in embryos injected with DMSO (A) or with 5 mM Cytochalasin D (B). Scale bars, 10 μm. (C-D) Rate of change in cell area in DMSO (C) or Cytochalasin D-injected embryos (D). Each line represents a single cell (n = 20 cells in 4 embryos in both C and D). (E) Oscillation amplitude for changes in cell area in embryos injected with DMSO (blue) or 5 mM Cytochalasin D (red). Asterisk indicates P < 0.05. Error
bars, s.e.m. (F-H) Vertex resolution during axis elongation in embryos expressing E-cadherin:GFP (green, top; grayscale, bottom) and myosin:mCherry (magenta, top) and injected with DMSO (F) or with 5 mM Cytochalasin D (G-H). Arrowheads indicate nascent DV interfaces. (I) Distribution of vertex resolution angles relative to the AP axis in embryos injected with DMSO (blue, $n = 50$ vertices in 5 embryos) or 5 mM Cytochalasin D (red, $n = 15$ vertices in 4 embryos). Angles were measured 150 s after the onset of vertex resolution. An angle of 90° with respect to the AP axis corresponds to the DV axis. (J) Length of new DV interfaces forming within 30° of the AP axis in embryos injected with DMSO (blue, $n = 43$ interfaces in 5 embryos) or 5 mM Cytochalasin D (red, $n = 9$ interfaces in 4 embryos). Time is with respect to the onset of vertex resolution, defined as the first time point in which the length of the nascent interface exceeded 1 µm. (I-J) Error bars, s.e.m. (K) Non-resolving vertex in an embryo expressing E-cadherin:GFP (green, top; greyscale, bottom) and myosin:mCherry (magenta, top), and injected with 5 mM Cytochalasin D. Arrowheads indicate the vertex. (A-B, F-H, K) Anterior left, dorsal up. (F-H, K) Scale bars, 5 µm.
If actomyosin contractility in the cells anterior and posterior to a resolving vertex drives directional interface assembly, then the nascent edge must be under tension. To quantify tension, we used an ultraviolet (UV) laser to locally irradiate and sever DV interfaces in embryos expressing E-cadherin:GFP, and particle-tracking velocimetry to quantify the change in position of the tricellular vertices once connected by the severed interface. The instantaneous retraction velocity of the vertices is proportional to the tension sustained by the interface prior to ablation (Hutson et al., 2003; Fernandez-Gonzalez et al., 2009). We compared retraction velocities after ablation of control DV junctions that were not actively elongating (average length of 7.3±0.3 µm, Figure 2.7A) and newly forming DV edges (average length of 3.4±0.2 µm, Figure 2.7B). The retraction velocity after ablation of new DV junctions was 0.81±0.08 µm/s, 32% greater than the retraction velocity after severing control DV edges (0.61±0.03 µm/s, P = 0.05, Figure 2.7C), indicating that—assuming uniform viscoelastic properties—new DV edges sustain increased mechanical tension with respect to non-elongating edges with similar orientation. New DV interfaces displayed smaller angles between the anterior or posterior cell junctions (θ<sub>avg</sub> = 136.6±3.3°) than control DV interfaces, (θ<sub>avg</sub> = 150.3±3.4°, P = 0.02, Figure 2.7A-B, D), and the retraction velocity after laser ablation was significantly anti-correlated with the angle between the anterior or posterior cell junctions (r = -0.6, P = 2.9x10^-5). Notably, no correlation was found between control or new DV interface length and instantaneous retraction velocity after ablation (r = 0.04 and 0.35, respectively, Figure 2.7E, F and Figure 2.8), suggesting that differences in retraction velocity between control and new DV edges are independent from interface length, and determined by whether the edge is being assembled. Vertex retraction after laser ablation could result from actomyosin contractility at the interface or at another structure (for example, another interface or a medial apical surface) connected to the severed interface. New DV edges were myosin-depleted (Blankenship et al., 2006) (P = 4.3x10^-5, Figure 2.9), suggesting that vertex retraction after ablation of new DV edges was caused by tension generated elsewhere and exerted onto the new edge. Together, our data strongly suggest that mechanical tension parallel to the AP axis of the embryo contributes to vertex resolution.
Figure 2.7: Resolving edges sustain increased mechanical tension during axis elongation.

(A-B) Germband cells expressing E-cadherin:GFP before and after ablation of a control DV edge (A) or a newly forming DV edge (B). White arrowheads point to the ablated interface. $\theta_1$ and $\theta_2$ indicate the angles between the junctions anterior and posterior to the ablated interface, respectively. Anterior left, dorsal up. Scale bars, 5 $\mu$m. (A'-B') Kymographs showing the vertex displacement caused by laser ablation of the edges shown in (A-B). Arrowheads indicate vertex position prior to ablation (green) or immediately after (yellow). Interfaces are rotated by 90° with respect to (A-B). Anterior down, dorsal left. Scale bar, 3 s.  

(C) Retraction velocity after laser ablation in control (blue, $n = 28$) and new (red, $n = 12$) DV interfaces. Asterisk indicates $P = 0.05$. Error bars, s.e.m.  

(D) Scatterplot showing interface length vs. average junction angle at the anterior and posterior ends ($\theta_{\text{avg}} = (\theta_1 + \theta_2)/2$).  

(E-F)
Scatterplots showing interface length vs. retraction velocity after laser ablation for control (E) and new (F) DV interfaces. Solid lines are best-fit lines.
Figure 2.8: The retraction velocity after ablation of new and control DV edges is not anti-correlated with their length.

(A-B) Kymographs showing the vertex displacements caused by laser ablation of relatively short (A, B) and long (A’, B’) control (A) or new (B) DV edges. Arrowheads indicate vertex position prior (green) or immediately after (yellow) ablation. Anterior down, dorsal left. Scale bars, 3 s.
Figure 2.9: New DV edges do not display a significant myosin accumulation.

(A-B) AP- (A) and newly forming DV- (B) oriented interfaces in embryos expressing E-cadherin:GFP (green) and myosin:mCherry (magenta). Arrowheads indicate the interfaces. Scale bars, 2 µm. Anterior left, dorsal up. (C) Myosin:mCherry fluorescence in AP and newly forming DV interfaces. Asterisks indicate $P < 0.001$. Error bars, s.e.m.
To further investigate the relative contribution of anterior/posterior and dorsal/ventral cells to new DV junction assembly during vertex resolution, we disrupted actomyosin contractility specifically in the anterior and posterior, or the dorsal and ventral cells. To this end, we used a UV laser to irradiate and destroy myosin networks in the cells anterior/posterior or dorsal/ventral to four-cell vertices. Cells expressed E-cadherin:GFP to visualize cell outlines, and myosin:mCherry to track the assembly of contractile networks. Cells were re-irradiated upon assembly of medial actomyosin networks to prevent the generation of contractile forces. Irradiated cells were not extruded in the course of these experiments. Controls were four-cell vertices in which the anterior/posterior or dorsal/ventral cell pairs were sham-irradiated with the UV laser fully attenuated using a neutral density filter. When the contractile activity of anterior/posterior cells was disrupted, 4/7 four-cell vertices did not resolve (their length was never greater than 1 µm for at least 1 min), in contrast to 0/10 vertices in sham-irradiated controls. In controls, the rate of new edge elongation calculated over 180 s was 0.47±0.08 µm/min (Figure 2.10A, C). Preventing contraction of the anterior/posterior cells resulted in a significant reduction of the rate of new edge elongation to 0.18±0.05 µm/min for the vertices that resolved (P = 0.03; Figure 2.10B-C). These results suggest that contractility in the cells anterior and posterior to a multicellular vertex is necessary for vertex resolution and the assembly of the new DV interface.

To investigate the role of dorsal/ventral cells in vertex resolution, we prevented assembly and contraction of medial actomyosin networks in the dorsal and ventral cells using laser ablation. In contrast with the ablation of anterior/posterior cells, ablation of the dorsal/ventral cells did not prevent vertex resolution: 5/7 new DV interfaces reached a length of at least 1 µm, similar to 10/10 in controls. The initial rates of elongation were similar, with new DV interfaces elongating at a rate of 0.37±0.11 µm/min over 60 s when contraction of the dorsal/ventral cells was disrupted, compared to rates of 0.50±0.15 µm/min in sham-irradiated controls (P = 0.48, Figure 2.10D-F). However, ablation of the dorsal and ventral cells resulted in a significant reduction of the rate of new interface elongation over the subsequent 120 s of elongation, from 0.52±0.11 µm/min in controls to -0.03±0.08 µm/min (P = 0.01, Figure 2.10D-F). Notably, in 3/5 vertices that resolved when dorsal/ventral cells were ablated, new DV edges formed but were not sustained beyond 1 min, collapsing back into
Figure 2.10: Local actomyosin contractility is necessary for vertex resolution and new DV interface assembly.

(A-B, D-E) Cells expressing E-cadherin:GFP (green) and myosin:mCherry (magenta) in sham-irradiated controls (A, D) or when UV irradiation was used to reduce local tension (B, E). White arrowheads indicate resolving interfaces. Asterisks show the targeted cells. Time is with respect to the first laser irradiation. Anterior left, dorsal up. Scale bars, 5 µm. (C, F) Length of resolving DV interfaces over time in controls (blue, n = 10 interfaces in C and F), under reduced AP tension (red, n = 7 interfaces in C), or under reduced DV tension (red, n = 7 interfaces in F). Discontinuities in the blue lines indicate times at which cells were targeted with the attenuated UV laser in all experiments. Error bars, s.e.m.
vertices. Together, our data suggest that dorsal/ventral cells are necessary to sustain the elongation of new DV interfaces, but not the resolution of multicellular vertices.

To determine if mechanical tension from the anterior and posterior cells can promote the elongation of new DV interfaces during germband extension, we developed a method to apply ectopic local tension to resolving vertices based on the wound healing response of germband cells (Campinho et al., 2013; Fernandez-Gonzalez and Zallen, 2013). Upon wounding by irradiation with a UV laser, germband cells undergo apical constriction driven by medial-apical actomyosin networks (Figure 2.11A). Apical constriction of germband cells generates ectopic tension on the surrounding cell interfaces (Figure 2.11A, arrowheads). We used a UV laser to wound the cells anterior and posterior to resolving vertices by irradiating their medial-apical surfaces (Figure 2.11B and Figure 2.12A-B). Under sham-irradiation (UV laser fully attenuated using a neutral density filter), the cell area and medial myosin of the anterior and posterior cells remained largely unaffected, and the new DV interface elongated at a rate of 0.79±0.14 µm/min (Figure 2.12A, C, E). Conversely, when the cells anterior and posterior were irradiated with UV light, myosin accumulated on the apical surface of the wounded cells and their apical areas decreased rapidly (Figure 2.12B, D), resulting in ectopic, AP-oriented tension on the resolving vertex. Under ectopic tension parallel to the AP axis, new DV junctions elongated at a rate of 1.73±0.30 µm/min, 2.1-fold faster than the elongation rate in controls ($P = 6.2 \times 10^{-3}$, Figure 2.12E). These results indicate that local mechanical tension parallel to the AP axis is sufficient to promote rapid assembly of new DV interfaces during vertex resolution in germband extension.

Our data that ectopic tension can increase the rate of new edge elongation suggest that tension parallel to the DV axis may change the direction of vertex resolution. We compared the orientation and rate of new edge elongation in sham-irradiated embryos (Figure 2.12F) and in embryos in which we induced apical constriction of the cells dorsal and ventral to a four-cell vertex, increasing tension along the DV axis (Figure 2.12G). All the four-cell vertices examined in control embryos ($n = 12$) resolved within 30° of the AP axis, and the rate of new interface assembly was 0.69±0.13 µm/min (Figure 2.12F,H). When we applied ectopic tension along the DV axis, the rate of new edge elongation was not affected.
Figure 2.11: Wounded cells undergo apical constriction and induce ectopic tension on adjacent cell-cell junctions.

(A-A’’) Germ band cells expressing E-cadherin:GFP (green, A’) and myosin:mCherry (magenta, A’’), before (pre-ablation) and at different times after UV-irradiation of the cell denoted by the yellow asterisk. White arrowheads indicate neighbouring junctions under ectopic strain when the wounded cell constricts apically. Time after wounding is shown. Anterior left, dorsal up. Scale bar, 5 µm. (B) Schematic representation of a method to induce ectopic AP-oriented tension (red arrows) on a vertex by wounding (yellow rays) the neighbouring anterior and posterior cells.
Figure 2.12: Local mechanical tension is sufficient to promote and orient new interface assembly during vertex resolution.

(A-B) Cells expressing E-cadherin:GFP (green) and myosin:mCherry (magenta) in sham (A) or UV-irradiated (B) embryos. (C-D) Medial myosin intensity (magenta) and cell area (green) in sham (C, n = 22 cells in 11 embryos) and UV-irradiated embryos (D, n = 16 cells in 8 embryos). (E) Length of resolving DV interfaces over time in controls (blue, n = 11 interfaces) and under increased tension along the AP axis (red, n = 8 interfaces). (F-G) Cells expressing E-cadherin:GFP in sham (F) or UV-irradiated (G) embryos. Asterisks show the cells around a four-cell vertex (white arrowheads) that were irradiated. Yellow arrowheads indicate the formation of a four-cell vertex. (A-B, F-G) Anterior left, dorsal up. Scale bars, 5 µm. (H) Length of resolving interfaces over time in controls (blue, n = 12) and under increased tension along the DV axis (red, n = 13). Turquoise indicates elongation parallel to the AP axis, pink denotes DV elongation. (C-E, H) Time is with respect to the time point when the nascent DV interface first exceeded 1 µm in length. Error bars, s.e.m. (C-D) Normalization is with respect to the value at 0 s.
(0.74±0.18 µm/min, P = 0.8), but the orientation of the new edge changed and occurred within 30° of the DV axis in 13 out of 13 cases (Figure 2.12G, H). Together, our data indicate that local tension can promote and orient the assembly of new cell-cell interfaces, suggesting a central role for mechanical forces during vertex resolution in *Drosophila* axis elongation.

### 2.5 Discussion

Polarized junction remodelling drives changes in tissue architecture from worms to mice (Walck-Shannon and Hardin, 2014). While junctional contraction and disassembly in the context of cell intercalation have been extensively explored (Bertet et al., 2004; Blankenship et al., 2006; Rauzi et al., 2008; Fernandez-Gonzalez et al., 2009; Levayer et al., 2011; Bosveld et al., 2012; Shindo and Wallingford, 2014; Lau et al., 2015), little is known about the mechanisms that control the directional assembly of new cell contacts during neighbour exchange. We used quantitative imaging, and biophysical and pharmacological approaches to show that local mechanical forces can direct the assembly of new junctions during *Drosophila* germband extension. New junctions elongate in pulses anti-correlated with the periodic contractions of the cells anterior and posterior to the new contact. Inhibiting actomyosin contractility disrupts both the rate and directionality of new junction assembly. Disrupting contractility in the cells anterior and posterior to the new edge disrupts vertex resolution and slows down new edge elongation, while preventing contraction of the dorsal and ventral cells mainly affects the maintenance and lengthening of the new cell interface. Hypercontraction of the cells anterior and posterior to the new edge accelerates the rate of new edge assembly. Finally, applying ectopic tension orthogonal to the characteristic orientation of vertex resolution is sufficient to alter the direction of new edge formation, suggesting that mechanical forces associated with actomyosin contractility direct the assembly of new cell contacts during multicellular vertex resolution in germband extension.

We show that vertex resolution occurs under increased mechanical tension, in a process that requires actomyosin contractility. Consistent with this, expression of inactive or constitutively active forms of myosin in embryos lacking the wild-type motor protein
disrupts the directionality of vertex resolution during germband extension (Kasza et al., 2014). Furthermore, mechanical tension is necessary for directional resolution of multicellular vertices in the mouse embryonic ectoderm during limb bud elongation (Lau et al., 2015). In the *Drosophila* dorsal thorax, whose architecture is determined by neighbour exchange events, actomyosin contractility in new edges is tightly regulated to facilitate their elongation (Bardet et al., 2013). Our data suggest that the increase in tension on the new contact may be caused locally by the pulsatile, anisotropic contraction of the cells around the resolving vertex. Interestingly, cells in the mouse limb bud ectoderm also display pulsed contractions that are disrupted in β-catenin mutants, and in these mutants the directionality of vertex resolution is lost (Lau et al., 2015). Together, these results are consistent with a general role for pulsed contractile activity in orienting and promoting cell intercalation.

We find that anterior/posterior and dorsal/ventral cells may play different roles during multicellular vertex resolution. Our data suggest that the anterior and posterior cells contribute to both vertex resolution and new edge elongation, while the dorsal and ventral cells are mainly necessary to support the elongation of the edge once the vertex has resolved. Recent mathematical modelling predicts that periodic actomyosin contractility in the medial-apical surface of anterior and posterior cells could drive the assembly of new edges during germband extension (Lan et al., 2015). The pulsed contraction of the anterior and posterior cells could cause rapid membrane reorganization in the dorsal and ventral cells (Pramanik et al., 2009), facilitating the assembly of an actin scaffold (Pickering et al., 2013) and the formation of junctions. Junctional and cytoskeletal remodelling require intact dorsal/ventral cells, and possibly, the continued stimulus from anterior/posterior cell pulsing. The implementation of optogenetic approaches (Guglielmi et al., 2015) to locally inhibit membrane remodelling and junctional and cytoskeletal dynamics will reveal how these processes are coordinated across cells to promote directional cell rearrangements during epithelial morphogenesis.

The mechanisms by which mechanical tension regulates the assembly of new cell interfaces during germband extension remain unclear. An accumulation of filamentous actin is the first known step of vertex resolution (Blankenship et al., 2006), and in this study, we found that
blocking actin polymerization results in multicellular vertices that do not resolve. Thus, actin polymerization may play a central role in vertex resolution. Mechanical forces can control actin dynamics \textit{in vitro}, possibly by inducing conformational changes in the formin family of actin regulators to favour faster and more frequent polymerization of actin filaments (Courtemanche et al., 2013; Higashida et al., 2013; Jegou et al., 2013). In addition, actin filaments are less susceptible to severing in the presence of increased tension (Hayakawa et al., 2011), which may accelerate actin assembly at nascent cell interfaces. Understanding how mechanical forces impact the localization and dynamics of different actin regulators will contribute to elucidating the mechanisms by which tension promotes directional cell behaviours during \textit{Drosophila} axis elongation.

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Chapter 3: Actomyosin cables prevent premature tissue internalization in the *Drosophila* embryo

3.1 Abstract
Compartment boundaries prevent mixing of cell populations during animal development. In the *Drosophila* embryo, the mesectoderm, a group of glial precursors that form the ventral midline, undergo oriented divisions during axis elongation and are eventually internalized approximately 6 hours later. Using spinning disk confocal microscopy and image analysis, we found that after cell division, mesectoderm cells reverse their polarity. As a consequence, the molecular motor non-muscle myosin II and its upstream activator Rho-kinase (Rok) accumulated at mesectoderm-ectoderm (ME) interfaces, forming supracellular cables flanking the mesectoderm on either side of the tissue. Laser ablation revealed the presence of increased tension at ME cables, where myosin was stabilized, as shown by fluorescence recovery after photobleaching. We used laser nanosurgery to reduce tension at the ME boundary, and we found that myosin fluorescence decreased rapidly, suggesting a role for tension in ME boundary maintenance. Surprisingly, the mesectoderm was prematurely internalized when we disrupted the ME cables mechanically or pharmacologically. Our results suggest that the redistribution of Rok following oriented cell divisions polarizes myosin and Baz within the mesectoderm to establish tissue boundaries, and that ME boundaries control the timely internalization of the mesectoderm as embryos develop.

3.2 Introduction
Compartment boundaries serve an essential function during animal morphogenesis. By restricting cells within compartments, tissue boundaries can maintain positional information within cell subgroups and ensure proper lineage specification (Langenberg and Brand, 2005; Voltes, 2018). Notably, compartment boundaries have important roles in cancer biology—
boundaries can isolate cancerous cells from surrounding epithelia, while their misregulation can result in tumour invasion and metastasis (Batlle et al., 2005; Cortina et al., 2007).

Mechanical forces play important roles in the establishment and maintenance of compartment boundaries. Actin and myosin are often enriched at boundaries, for instance, at the anterior-posterior compartment boundary in the wing disc (Landsberg et al., 2009; Umetsu et al., 2014a), the epidermal parasegmental boundaries (Monier et al., 2010; Tetley et al., 2016; Scarpa et al., 2018; Urbano et al., 2018), and around precursor salivary gland placode cells (Sanchez-Corrales et al., 2018) in Drosophila; at the mesoderm-ectoderm boundary (Rohani et al., 2011) and between the notochord and presomitic mesoderm (Fagotto et al., 2013) in Xenopus; between germ layers (Krieg et al., 2008), around the eye field (Cavodeassi et al., 2013), and at the borders delimiting hindbrain segments (Calzolari et al., 2014) in zebrafish; and within the neuroepithelium in mice (O'Neill et al., 2016). Cytoskeletal contractility maintains tissue boundaries over time, both in vivo (Landsberg et al., 2009; Aliee et al., 2012; Tetley et al., 2016; Urbano et al., 2018) and in vitro (Javaherian et al., 2015), and increased cell mixing emerges upon boundary loss (Major and Irvine, 2006; Landsberg et al., 2009; Monier et al., 2010; Calzolari et al., 2014). In particular, in the Drosophila embryo, cell divisions can challenge parasegmental boundaries, but myosin-based contraction of the boundary keeps the two daughter cells within the body segment from which they originate (Monier et al., 2010). Thus, actomyosin contractility is able to provide the means necessary to restrict cell groups from intermixing; however, little is known about how boundaries form and how they are maintained in vivo.

In Drosophila, the mesectoderm separates the ventral mesoderm from the lateral ectoderm, forming the ventral midline of the animal after the mesoderm is internalized. During axis elongation (approximately 3.5 hours after egg laying), the Drosophila ectoderm extends by more than twofold along the anterior-posterior axis of the animal (Irvine and Wieschaus, 1994). Axis elongation is driven by the planar-polarized distribution of actin, myosin and the upstream myosin activator, Rho-kinase, which preferentially localize to cell interfaces between anterior and posterior (AP) neighbours in both the ectoderm and the mesectoderm. Complementary to this, the polarity factor Bazooka/Par-3 and adherens junction components
localize to interfaces between dorsal-ventral (DV) neighbours (Zallen and Wieschaus, 2004; Blankenship et al., 2006; Simoes et al., 2010). At the final stages of axis elongation (4.5 hours after egg laying), mesectoderm cells divide directionally along the anterior-posterior axis to reduce the strain generated as the adjacent ectoderm extends (Wang et al., 2017). Eventually, mesectoderm cells are also internalized (10 hours after egg laying), giving rise to glia and neurons of the central nervous system (Jacobs and Goodman, 1989; Klambt et al., 1991; Tepass and Hartenstein, 1994; Wheeler et al., 2006). However, the mechanisms that maintain the separation between the mesectoderm and the ectoderm remain unclear.

Here, we explore the establishment and maintenance of tissue boundaries at the mesectoderm-ectoderm (ME) interface. Using quantitative live microscopy, we found that a reversal in the polarity of mesectoderm cells after division resulted in the enrichment of myosin specifically at the ME boundary, forming supracellular cables on either side of the mesectoderm. Supracellular myosin cables sustained increased tension and exhibited reduced myosin turnover, suggesting that tension regulates the dynamics of myosin at the ME boundary. Consistent with this, myosin levels at the ME boundary decreased when we mechanically reduced tension at the ME interface. Strikingly, mechanical disruption of the cable was also associated with premature internalization of the mesectoderm. Similarly, pharmacological inhibition of myosin-based contraction disrupted the myosin cable at the ME boundary and allowed ectoderm cells to divide into the mesectoderm, again leading to the early internalization of the mesectoderm. Together, our data reveal that ME boundaries arise through a reversal in mesectoderm cell polarity, promoting the assembly of supracellular myosin cables that generate tensile stress and resist cell mixing across tissue compartments.

3.3 Materials and methods

3.3.1 Fly stocks

We used the following markers for live imaging: UASp-baz:GFP (Benton and St Johnston, 2003), UAS-baz:mCherry (McGill et al., 2009) (Bloomington Drosophila Stock Center #65844), sqh-gap43:mCherry (Martin et al., 2010), sqh-GFP:moesinABD (Kiehart et al.,
2000), sqh-GFP:rok\textsuperscript{K116A} (Simoes et al., 2010), sqh-sqh:GFP (Royou et al., 2004), sqh-sqh:mCherry (Martin et al., 2009), and UAS-sqh:GFP (gift of E. Caussinus). baz:mCherry and baz:GFP embryos were the F2 progeny of UAS males crossed to matatub67 and matatub67;15 females, respectively (gifts of J. Zallen and S. Simoes). sqh:GFP embryos were the F1 progeny of UAS males crossed to sim-GAL4 females (Xiao et al., 1996) (Bloomington Drosophila Stock Center #9150).

3.3.2 Time-lapse imaging

Stage 7-9 embryos were dechorionated in 50% bleach for 2 min, rinsed, glued ventral side down to a glass coverslip using heptane glue, and mounted in a 1:1 mix of halocarbon oil 27 and 700 (Sigma-Aldrich, St. Louis, MO). For single-interface ablations, a YSI membrane was placed on top of the glass coverslip. Embryos were imaged using a Revolution XD spinning disk confocal microscope equipped with an iXon Ultra 897 camera (Andor, Belfast, UK) and a 1.5x coupling lens. Single-interface ablations, FRAP, and protrusion imaging were done using a 100x oil immersion lens (Olympus, Shinjuku, Japan; NA 1.40); the effects of drug treatments were imaged with a 60x oil immersion lens (Olympus, NA 1.35); for all other experiments, a 40x oil immersion lens (Olympus, NA 1.35) was used. Sixteen-bit Z-stacks were acquired at 0.2-0.3 µm steps every 30-60 s (11-17 slices per stack), and maximum intensity projections were used for analysis.

3.3.3 Laser ablation

Ablations were induced using a pulsed Micropoint N\textsubscript{2} laser (Andor) tuned to 365 nm. The laser delivers 120 µJ pulses of 2-6 ns each. For ablation of cell boundaries, 10 consecutive laser pulses were delivered to a single spot along a cell interface. For cuts across the mesectoderm, 10 consecutive pulses were delivered at discrete points ~1.7 µm apart along a 30 µm line. Line ablations were performed after the last mesectoderm division, when myosin cables had formed at the interface between ectoderm and mesectoderm. For control experiments, the laser was fully attenuated using a neutral density filter.
3.3.4 Fluorescence Recovery After Photobleaching (FRAP)

Photobleaching experiments were conducted using the FRAPPA system (Andor) and a 488 nm laser. A 10x10 pixel region (1.1x1.1 µm$^2$) was photobleached using a laser dwell time of 500 µs/pixel. Two Z-stacks were acquired 3 seconds apart immediately prior to photobleaching. Regions were imaged immediately after photobleaching every 3 seconds for at least 2 minutes. Fluorescence intensity in the photobleached region at time $t$, $f(t)$, was measured as:

$$f(t) = 100 \times \frac{(I_{ROI}(t) - I_{bg}(t)) - (I_{ROI}(0) - I_{bg}(0))}{(I_{ROI}(before) - I_{bg}(before)) - (I_{ROI}(0) - I_{bg}(0))} \times \frac{(I_M(before) - I_{bg}(before))}{(I_M(t) - I_{bg}(t))},$$  \hspace{1cm} (3.1)

where $t = before$ represents the timepoint immediately before photobleaching; $t = 0$ is the timepoint immediately after photobleaching; $I_{ROI}$ is the mean pixel value within the photobleached region; $I_{bg}$ is the background signal, calculated as the most frequent pixel value (the mode) of a single image slice approximately 2-3 microns underneath the apical-most surface of the embryo; and $I_M$ is the mean image intensity. The mobile fraction was calculated as the mean $f(t)$ value for the last two time points measured, and the half time ($t_{1/2}$) was the time required to reach half of the mobile fraction.

3.3.5 Drug injections

Embryos were dechorionated and glued to a coverslip as above, dehydrated for 15 minutes, and covered with a 1:1 mix of halocarbon oil 27 and 700 (Sigma-Aldrich). Embryos were injected using a Transferman NK2 micromanipulator (Eppendorf, Hamburg, Germany), and a PV820 microinjector (WPI, Sarasota, FL) attached to the spinning disk confocal microscope. Drugs (Y-27632, Tocris Bioscience, Bristol, UK and H-1152, Tocris Bioscience, Bristol, UK) were injected into the perivitelline space, where they are predicted to be diluted 50-fold (Foe and Alberts, 1983). Y-27632 and H-1152 were injected at 10 mM in water; control embryos were injected with water. Embryos were imaged immediately before injection and after, for at least 60 minutes.
3.3.6 Cell segmentation and image analysis

Image analysis was performed using algorithms developed with Matlab (MathWorks, Natick, MA) and DIPImage (Delft University of Technology, Delft, Netherlands) and integrated in our custom Scientific Image Segmentation and Analysis (SIESTA) software (Fernandez-Gonzalez and Zallen, 2011; Leung and Fernandez-Gonzalez, 2015).

To delineate interfaces and cables of interest, we used the semi-automated LiveWire algorithm implemented in SIESTA (Fernandez-Gonzalez and Zallen, 2013). In short, the user clicks on a pixel on the edge of the cell to be segmented, and as the mouse moves the brightest set of pixels connecting the first pixel and the current position of the mouse is calculated using Dijkstra’s minimal cost path algorithm (Dijkstra, 1959). Subsequent mouse clicks along the boundary freeze the trajectory and reinitiate the search from the last clicked point. Mean fluorescence was measured from maximum intensity projections, and background-subtracted using the most frequent pixel value (the mode) of a single image slice approximately 2-3 microns underneath the apical-most surface of the embryo. Intensity values were corrected for photobleaching by dividing by the mean intensity of the maximum intensity projection in each time point.

For polarity measurements, we used LiveWire to determine fluorescence values and angular orientation (0-90°) of each interface. For each embryo, cell interfaces were grouped into 15°-bins and normalized to the mean intensity of edges parallel (0°-15°) or perpendicular (75°-90°) to the AP axis.

To characterize ME boundaries following large ablations or drug injections, we used LiveWire to delineate both boundaries within the center-most 150 pixels of the image. We determined the mean myosin intensity along the 150-pixel segment of each cable for every timepoint as described above, and we calculated the mean width of the mesectoderm by determining the distance between pixel pairs across both boundaries. We normalized myosin levels to the intensity of the cable in the timepoint immediately prior to ablation or injection, and we defined the rate of change in myosin fluorescence as:
rate of change in myosin fluorescence (%) = \(100\% \ast (m(t) - m(t_0))\), \hspace{1cm} (3.2)

where \(m(t)\) represents myosin intensity of the ME boundary at time \(t\), and \(t_0\) is the time immediately following ablation or injection. Similarly, we normalized width values to the mesectoderm width in the timepoint immediately prior to ablation or injection, and we defined the rate of change in mesectoderm width as:

rate of change in mesectoderm width (%) = \(100\% \ast (w(t) - w(t_0))\), \hspace{1cm} (3.3)

where \(w(t)\) represents mean mesectoderm width at time \(t\), and \(t_0\) is the time immediately following ablation or injection.

3.3.7 Statistical analysis

Sample means were compared using a non-parametric Mann–Whitney test (Glantz, 2002). For comparisons of more than two groups, we used a Kruskal–Wallis test to reject the null hypothesis, and Dunn's test for pairwise comparisons. For time series, error bars indicate the standard error of the mean (s.e.m.). For box plots, error bars show standard deviation, the box indicates the s.e.m., and grey lines denote the mean.

3.4 Results

3.4.1 Mesectoderm cells reverse their polarity after division

We investigated the presence of a boundary at the interface between ectoderm and mesectoderm cells. To this end, we used spinning disk confocal microscopy to image stage 7 embryos (3.5 hours old) expressing a fluorescent membrane reporter. We found that, following oriented cell divisions, mesectoderm cells were packed into a characteristic conformation that rendered the mesectoderm morphologically distinct from the adjacent ectoderm (Figure 3.1A). The interface between mesectoderm and ectoderm became increasingly linear over time, suggesting that ectoderm and mesectoderm are separated by a boundary and do not mix.
Figure 3.1: Myosin, Bazooka, and Rok reverse their polarity after mesectoderm cell divisions.

(A) Mesectoderm cells in an embryo expressing Gap43:mCherry, a membrane marker. Yellow dotted lines delineate the mesectoderm. Cyan boxes indicate the regions shown in (A’). Time is with respect to completion of the first mesectoderm division (arrowhead). Scale bars, 50 µm (A) and 10 µm (A’). (B-C, F-G) Mesectoderm cells in an embryo expressing myosin:GFP (B-C, green; B’-C’) and Bazooka:mCherry (B-C, magenta; B’’-C’’), or Rok:GFP (F-G, green; F’-G’) and myosin:mCherry (F-G, magenta; F’’-G’’), 5 min before (B, F) and 20 min after (C, G) the first mesectoderm division. Scale bars, 10 µm. (D-E, H-I) Mean myosin (D) and Bazooka (E), and Rok (H) and myosin (I) fluorescence in mesectoderm cell interfaces binned according to their orientation, 5 min before (blue) and 20 min after (red) the onset of cell division (n = 4 embryos in D-E; n = 3 in H-I). Error bars denote s.e.m. (A, B-C, F-G) Anterior left.
Tissue boundaries are associated with the presence of supracellular cables formed by actin and the motor protein non-muscle myosin II (Major and Irvine, 2006; Krieg et al., 2008; Landsberg et al., 2009; Monier et al., 2010; Rohani et al., 2011; Cavodeassi et al., 2013; Fagotto et al., 2013; Calzolari et al., 2014; Umetsu et al., 2014a; O’Neill et al., 2016; Tetley et al., 2016; Sanchez-Corrales et al., 2018; Scarpa et al., 2018; Urbano et al., 2018). In the *Drosophila* ectoderm, myosin forms cables parallel to the dorsal-ventral axis of the embryo that act as boundaries to prevent cell mixing across body segments (Monier et al., 2010). To investigate if a myosin cable separates ectoderm and mesectoderm, we imaged embryos expressing a GFP-tagged form of the myosin regulatory light chain, encoded by the *Drosophila* gene *spaghetti squash* (*sqh*). We found that, before the onset of oriented divisions in the mesectoderm that facilitate axis elongation (Wang et al., 2017), myosin localized preferentially to AP junctions in mesectoderm cells, similar to the pattern observed in the ectoderm (Zallen and Wieschaus, 2004). In the mesectoderm, AP interfaces displayed a 1.2-fold increase in myosin fluorescence with respect to interfaces between dorsal and ventral cells (DV interfaces) (*P* < 0.05, Figure 3.1B, D). Consistent with this, we found no evidence of a myosin cable at the interface between ectoderm and mesectoderm (Figure 3.1B).

Strikingly, mesectoderm cells became repolarized after cell division: when we imaged the distribution of myosin 20 min after the first mesectoderm cell division, we found that myosin became enriched at DV interfaces and was depleted from AP junctions. Mesectoderm cells displayed 1.3-fold greater fluorescence in DV than in AP interfaces (*P* < 0.05, Figure 3.1C-D). As a consequence, we found that a myosin cable was formed on either side of the mesectoderm, at the boundary with the ectoderm. Together, these data show that the planar polarity of myosin in the mesectoderm is reversed after cell division, resulting in the assembly of a myosin cable, a supracellular network connected across cells at adherens junctions, at the mesectoderm-ectoderm (ME) interface that may act as a boundary between the two tissues. Experiments in which myosin:GFP was specifically expressed in mesectoderm confirm that the cable exists at least partially through mesectodermal contribution (Figure 3.2, middle).
Figure 3.2: ME cables are at least partially comprised of a mesectoderm-specific myosin population.

Myosin populations visualized through the expression of myosin:mCherry expressed throughout the embryo (top, magenta; bottom, greyscale) and UAS-myosin:GFP expressed specifically in the mesectoderm under the sim-GAL4 driver (top, green; middle, greyscale). Scale bars, 10 µm. Anterior left.
To further investigate the mechanisms by which myosin polarity is reversed in the mesectoderm, we quantified the distribution of other proteins known to regulate myosin polarity. The establishment of AP myosin cables in the ectoderm requires the activity of the polarity factor Bazooka/Par-3. In the ectoderm, Bazooka localizes preferentially to DV junctions and excludes myosin from those interfaces (Zallen and Wieschaus, 2004; Blankenship et al., 2006; Simoes et al., 2010). To elucidate whether Bazooka may be important to repolarize mesectoderm cells after their division, we imaged embryos co-expressing mCherry-tagged Bazooka and myosin:GFP (Figure 3.1B-C). Before cell division, mesectoderm cells displayed a distribution of Bazooka consistent with that of the ectoderm: Bazooka fluorescence was 1.2-fold greater in DV than AP interfaces ($P < 0.05$, Figure 3B, E). Following cell division, we found that Bazooka also reversed its polarity in the mesectoderm, and became enriched in AP junctions, with fluorescence levels 1.4-fold greater than those at DV junctions ($P < 0.05$, Figure 3.1C, E). Together, these data show that both myosin and Bazooka invert their distribution in mesectoderm cells after cell division, suggesting that the assembly of myosin cables at the ME boundary may result from a general reversal of mesectoderm cell polarity.

In the ectoderm, Bazooka polarity is established by the upstream myosin activator Rho-kinase (Rok) (Simoes et al., 2010). Rok localizes to AP junctions in the germband, where it phosphorylates and displaces Bazooka from the junction. To investigate if Rok may be important for the repolarization of the mesectoderm after cell division and the establishment of the myosin cable at the interface with the ectoderm, we imaged embryos expressing myosin:mCherry and Rok:GFP. After cell division, Rok preferentially localized to DV interfaces in the mesectoderm, with an enrichment of 1.3-fold with respect to AP interfaces (Figure 3.1F-I), suggesting that Rok could control the localization of Bazooka and myosin during the repolarization of mesectoderm cells. Together, our data suggest that a polarity system including Rok, Bazooka, and myosin establishes tissue boundaries separating ectoderm from mesectoderm following the completion of mesectoderm cell divisions.
3.4.2 ME cables sustain increased tension and exhibit reduced myosin turnover

Tissue boundaries characteristically sustain increased tension to resist cell mixing, as demonstrated in the compartment boundaries within the *Drosophila* wing disc (Landsberg et al., 2009; Alix et al., 2012) and in the boundaries separating body segments in the *Drosophila* ectoderm (Tetley et al., 2016; Urbano et al., 2018). We used laser ablation to investigate the tension sustained by ME cables. To determine whether ME cables sustained increased tension, we severed individual segments of the cable in embryos expressing myosin:GFP, and we compared to myosin-depleted mesectoderm AP interfaces (Figure 3.3A-B). As an additional control, we also ablated myosin-enriched ectoderm AP interfaces (Figure 3.3C), which are under high levels of tension as cells intercalate (Fernandez-Gonzalez et al., 2009). We measured the recoil velocity after ablation as an estimate of edge tension (Hutson et al., 2003; Fernandez-Gonzalez et al., 2009). On average, the ME boundary displayed recoil velocities 2.0 times faster than AP interfaces in the mesectoderm, while ME boundary retraction was comparable to that of AP interfaces in the ectoderm ($P < 0.0001$ and $P > 0.05$, respectively, Figure 3.3D). Furthermore, the initial retraction velocity after ablation correlated significantly with myosin fluorescence prior to ablation ($r = 0.7$, $P = 1.5 \times 10^{-16}$, Figure 3.3E). Together, these data indicate that myosin enrichment promotes tensile stress along the ME boundary, consistent with a role for the myosin cable in segregating ectoderm from mesectoderm.

Myosin dynamics can be regulated by tensile stress, both *in vitro* (Kovacs et al., 2007) and *in vivo* (Effler et al., 2006; Fernandez-Gonzalez et al., 2009; Pouille et al., 2009; Kobb et al., 2017). To establish whether differences in tension affect myosin dynamics at the ME boundary, we used Fluorescence Recovery After Photobleaching (FRAP) in embryos expressing myosin:GFP to compare myosin turnover at the ME boundary or in AP junctions in the mesectoderm (Figure 3.3F-H). We measured two parameters in FRAP experiments: the mobile fraction and the half time of fluorescence recovery. The mobile fraction quantifies the amount of protein turning over. There was no difference in mobile fraction between AP junctions and ME boundary segments ($P > 0.20$, Figure 3.3I), nor was there a relationship between mobile fraction and initial myosin fluorescence ($r = 0.03$, $P = 0.85$), suggesting that the myosin enrichment does not affect the amount of myosin that turns over. In contrast, the half time of fluorescence recovery, which is indicative of the rate of myosin turnover, was significantly longer at the ME boundary:
Figure 3.3: The ME boundary sustains increased tension and displays myosin stabilization.

(A-C) Embryos expressing myosin:GFP immediately before and after ablation of an AP junction in the mesectoderm (A), a segment of the myosin cable at the ME boundary (B), or an AP junction in the ectoderm (C). White arrowheads point to the interface prior to ablation. Scale bars, 5 µm. (A’-C’) Kymographs showing the displacements caused by laser ablation of the edges shown in (A-C). Arrowheads indicate vertex position prior to ablation (cyan) or immediately after (red). Scale bar, 4 s. (D-E) Recoil velocity after ablation with respect to pre-ablation myosin fluorescence (E), and in mesectoderm AP interfaces (blue, n = 27 interfaces), ME boundary segments (red, n = 35), and ectoderm AP interfaces (black, n = 32).
Best-fit line in grey. (F-G) Kymographs showing FRAP experiments in embryos expressing myosin:GFP on mesectoderm AP interfaces (F, blue) or segments of the ME boundary cable (G, red). Boxes indicate the photobleached region. Green dashed lines show the time of photobleaching. Scale bars, 3 s. (H-J) Percent pre-bleach fluorescence over time in the photobleached region (H), mobile fraction (I), and half time (J) for mesectoderm AP interfaces (blue, n = 17 interfaces) and ME boundary segments (red, n = 22 interfaces). (A-C, F) Anterior left. (B’, G) Anterior down. (H) Error bars, standard error of the mean (s.e.m.). (D, I, J) For box plots, error bars show the s.d., the box indicates the s.e.m., and grey lines denote the mean.
myosin turned over with a characteristic half time of 23 ± 5 s at the ME boundary, significantly slower than the 7 ± 1 s at AP interfaces \((P < 0.001, \text{Figure 3.3F-G,K})\). There was a significant correlation between the half time of fluorescence recovery and the initial myosin fluorescence \((r = 0.5, \ P = 0.004)\), suggesting that higher levels of tension further stabilizes myosin. Together, our data show that myosin is stabilized at the ME boundary, suggesting that the increase in tension caused by myosin-based contraction may contribute to boundary maintenance through a reduction in the rate of myosin turnover.

3.4.3 Tension stabilizes myosin at the ME boundary
To test whether tension at the ME boundary plays a role in myosin stabilization, we reduced the tension sustained by ME cables by making 30-μm long incisions across the mesectoderm at the anterior and posterior ends of the tissue (Figure 3.4A-B). In newly formed ME cables, approximately 60 min after the first mesectoderm division, myosin at the boundary stayed relatively constant in sham-irradiated controls, decreasing by 3.0±2.7% over the initial ten minutes, with no obvious change in the width of the mesectoderm (a 0.1±3.3% decrease) (Figure 3.4A, C-D). Subsequently, myosin fluorescence at the cable decreased in parallel with a reduction in the width of the mesectoderm as it was internalized (Figure 3.4A, C-D). Thirty minutes after the assembly of the ME cable, myosin levels decreased by 22.1±2.5% \((P < 0.001, \text{Figure 3.4C})\), and the mesectoderm was 14.4±4.2% narrower \((P < 0.02, \text{Figure 3.4D})\), with respect to the maxima. Together, these data suggest that disassembly of the ME cable may facilitate mesectoderm internalization during Drosophila embryonic development.

Reducing tension at the ME boundary affected myosin dynamics at the cable and the internalization of the mesectoderm. When we reduced boundary tension at new ME cables, myosin levels decreased by 21.5±3.6% over the initial 10 min \((P < 0.002, \text{Figure 3.4C})\). Concomitantly, over 30 minutes, the width of the mesectoderm decreased by 34.4±2.6%, 2.4 times faster than in control embryos \((P < 0.002, \text{Figure 3.4D})\). These results indicate that tension is at least in part necessary to maintain the myosin cable at the ME boundary, and that the cables may play a role in maintaining the mesectoderm on the surface of the embryo prior to its internalization.
Figure 3.4: Tension regulates myosin dynamics at the ME boundary and mesectoderm position.

(A-B,E-F) Mesectoderm cells expressing myosin:GFP before and after sham irradiation (A,E) or laser incisions at the anterior and posterior ends of the tissue (B,F), immediately after ME boundary cable formation (A-B) or 90 minutes later (E-F). Time is with respect to laser irradiation. Yellow dotted lines delimit the ME boundaries. Magenta dotted lines indicate regions targeted by the laser. Scale bars, 10 µm. Anterior left. (C-D, G-H) Relative myosin fluorescence at ME cables (C, G) and mesectoderm width (D, H) in sham-treated controls (blue, n = 22 cables in 11 embryos in C and D, and n = 20 cables in 10 embryos in G and H), or in laser-irradiated embryos (red, n = 20 cables in 10 embryos in C and D, and n = 20 cables in 10 embryos in G and H). Error bars, s.e.m.
To investigate if tension contributes to the dynamics of the ME cable once the mesectoderm is being internalized, we reduced tension in the mesectoderm 120 minutes after cable formation (Figure 3.4E-F). Reducing tension did not affect the levels of myosin at the ME boundary, which decreased by 11.7±2.0% over 30 minutes in sham-treated controls, and by 17.4±2.8% in laser-irradiated embryos ($P > 0.10$; Figure 3.4G). Similarly, the width of the mesectoderm continued to decrease at the same rate in controls and laser-irradiated embryos ($P > 0.20$, Figure 3.4H). Together, these data indicate that tension does not affect myosin levels at the ME boundary after mesectoderm internalization has begun.

3.4.4 Rok maintains the boundary between ectoderm and mesectoderm

In control embryos, mesectoderm internalization is coincident with the decrease of myosin at ME cables (Figure 3.4C-D, G-H, blue), suggesting that myosin contractility may influence the timing of tissue internalization. To further characterize this, we quantified myosin fluorescence at ME cables, as well as mesectoderm width, from the first appearance of the cables until the tissue was internalized completely (Figure 3.5). Mesectoderm width decreased gradually, and internalization was fully complete at the end of germband retraction, in which segments of the mesectoderm corresponding to each thoracic parasegment were internalized (Figure 3.5, yellow brackets). Importantly, mesectoderm internalization occurred alongside a gradual decrease in myosin at ME cables. Mean myosin intensity spiked as germband retraction progresses, and myosin concentration increased until internalization was complete. These data suggest that myosin could be required for the long-term maintenance of ME cables.

To further establish if the myosin cables at the ME boundaries control the timing of mesectoderm internalization, we disrupted myosin contractility by treating embryos with 10 mM of Y-27632, a Rok inhibitor (Uehata et al., 1997; Narumiya et al., 2000) (Figure 3.6A-B). Embryos were injected approximately 60 minutes after the onset of cell division, when the ME cables had been established. In water-injected embryos, Bazooka polarity was unaffected. Water injections resulted in a 17.7±3.0% reduction in myosin fluorescence at the ME boundary cable over the course of 20 minutes, without compromising the integrity of the
Figure 3.5: Myosin density throughout the progression of mesectoderm internalization.

(A) Mesectoderm cells in an embryo expressing myosin:GFP and membrane:mCherry. Yellow brackets denote separate mesectoderm segments that appear as the germband retracts during the internalization process. Time is in hours post-fertilization (hpf). Scale bars, 50 µm. Anterior left. (B) Mesectoderm width (blue) and mean myosin intensity at ME cables (red) as measured from the end of oriented mesectoderm divisions until the completion of tissue internalization.
Figure 3.6: Y-27632 treatment disrupts the myosin cable at the ME boundary.

(A-B) Mesectoderm cells in embryos expressing membrane:mCherry (A-B, magenta; A’-B’, greyscale) and myosin:GFP (A-B, green; A’’-B’’, greyscale), and injected with water (A) or 10 mM Y-27632 (B). (C) Reduction in myosin fluorescence at ME boundary 20 mins after injection of water (blue, n = 20 boundaries in 10 embryos) or 10 mM Y-27632 (red, n = 20 boundaries in 10 embryos). Scale bars, 10 μm. Anterior left.
boundary: ectoderm cells were not able to divide into the mesectoderm, and the ME boundary remained straight (Figure 3.7A). The mesectoderm was not completely internalized during the 90 minutes following injection (Figure 3.7A, C, E). In contrast, in embryos treated with Y-27632, Bazooka polarity was abolished (Figure 3.7F-G), and myosin fluorescence at the ME boundary decreased by 57.1±2.6% over 20 minutes, a significantly greater reduction than in water-treated controls (Figure 3.6C), confirming the disruption of myosin activity by Y-27632. As a consequence, ectoderm cells were able to divide into the mesectoderm (Figure 3.7B; see yellow numbers marking daughter ectoderm cells), and the linearity of the ME boundary decreased rapidly over time. Thus, when we inhibited Rok, mesectoderm width decreased at a rate 1.4-fold faster than in controls ($P < 0.05$; Figure 3.7E), resulting in complete internalization of the tissue within 90 min of Rok inhibition. The premature internalization of the mesectoderm was associated with ectoderm cells taking over the space previously occupied by the mesectoderm (Figure 3.7D-E). Together, these data suggest the ME boundary cables may counteract the premature internalization of the mesectoderm by preventing ectoderm cells from dividing into the mesectoderm territory.

The premature internalization of the mesectoderm upon ME boundary disruption could also be linked to increased cell motility. Consistent with this, Y-27632 treatment in embryos expressing membrane:mCherry and GFP:MoesinABD (actin binding domain), a reporter for filamentous actin (Kiehart et al., 2000) resulted in a dramatic increase in protrusive activity, both in ectoderm and mesectoderm cells (Figure 3.8A-B), suggesting a mode by which ectoderm cells could spread over the mesectoderm. These data strongly suggest that Rok-induced tension at the ME boundary controls the internalization of the mesectoderm by establishing a tissue boundary that prevents the spread of the ectoderm.

### 3.5 Discussion

Boundaries compartmentalize tissues to ensure appropriate patterning and cell fate specification. Here, we used quantitative imaging to characterize how tissue boundaries are established in the early *Drosophila* embryo. In the mesectoderm, a reversal in cell polarity results in the formation of supracellular myosin cables which segregate the mesectoderm
Figure 3.7: Rok inhibition results in premature mesectoderm internalization.

(A-B) Mesectoderm cells in an embryo expressing membrane:mCherry (A-B, magenta; A’-B’, greyscale) and Bazooka:GFP (A-B, green; A’’-B’’, greyscale), injected with water (A) or 10 mM Y-27632 (B). White and cyan numbers track randomly selected mesectoderm and ectoderm cells, respectively. Yellow numbers indicate one or both daughters of a tracked
ectoderm cell following division. Time is with respect to the first timepoint acquired after injection. Scale bars, 10 µm. Anterior left. (C-D) Mesectoderm cells in an embryo expressing membrane:mCherry (magenta) and Bazooka:GFP (green), injected with water (C) or 10 mM Y-27632 (D). Yellow dotted lines indicate location of XZ panels. Cyan asterisks mark ectoderm cells. Black arrowheads delimit the mesectoderm. White arrowheads indicate mesectoderm cells. Time is with respect to the first timepoint acquired after injection. Scale bars, 10 µm (XY) and 1 µm (XZ). Anterior down (XY). (E) Relative mesectoderm width in embryos injected with water (blue, n = 3 embryos) or 10 mM Y-27632 (red, n = 7). (F-G) Mean Bazooka fluorescence in mesectoderm interfaces binned according to their orientation, immediately prior to and 20 min following injection of water (blue, n = 10 embryos) or 10 mM Y-27632 (red, n = 9 embryos).
Figure 3.8: Rok inhibition results in upregulated protrusive activity.

(A-B) Mesectoderm cells in an embryo expressing membrane:mCherry (F-G, magenta) and GFP:actin (A-B, green; A’-B’, greyscale), injected with water (A) or 10 mM Y-27632 (B). Yellow dotted lines delimit the ME boundaries. Scale bars, 10 µm. Anterior left.
from the ectoderm. Increased tension at the boundary prevents cell mixing and contributes to tissue maintenance by stabilizing myosin. Mechanical and pharmacological cable disruption results in the premature internalization of the mesectoderm. Thus, our findings demonstrate that myosin cables can maintain tissue position while acting as boundaries that prevent cell mixing.

We introduce the interface between mesectoderm and ectoderm as a new model to study compartment boundaries. The ME boundary forms rapidly, is accessible to live imaging, and amenable to genetic, pharmacological, and biophysical manipulations. Using live imaging we provide evidence for the cellular mechanisms of boundary formation, which are linked to a reversal in the polarity of mesectoderm cells following division. The mechanisms that trigger the inversion in the distribution of Rok, myosin and Bazooka in mesectoderm cells remain unclear. Bazooka localizes to nascent cell contacts during cell intercalation in the Drosophila ectoderm (Blankenship et al., 2006), and after oriented cell divisions in the Drosophila pharynx (Tamada and Zallen, 2015), presumably to stabilize the newly forming junctions. Bazooka can bind to β-catenin, a member of the adherens junction complex (Wei et al., 2005), and Bazooka activity is required for proper apical localization of adhesion complexes (Muller and Wieschaus, 1996; Harris and Peifer, 2004). Thus, it is possible that Bazooka is recruited to new interfaces after mesectoderm cell division to support the formation of new adherens junctions, and that Bazooka repolarization is a consequence of new junctions being AP interfaces, as mesectoderm divisions are biased towards the anterior-posterior axis of the embryo (Wang et al., 2017). Myosin localization depends on Bazooka polarity in the ectoderm (Simoes et al., 2010), and thus Bazooka could control myosin polarity in the mesectoderm, and hence the formation of ME boundaries. Further studies investigating the interdependence of protein redistribution following divisions, as well as protein dynamics if cell divisions are inhibited in the mesectoderm, will help to clarify the degree of involvement that Bazooka and cell division have in establishing mesectoderm polarity.

Our results show that a tension-bearing supracellular actomyosin cable forms a boundary between ectoderm and mesectoderm. Previous studies demonstrated that actomyosin
contractility is a primary feature of tissue boundaries, both in vivo (Landsberg et al., 2009; Aliie et al., 2012; Tetley et al., 2016; Urbano et al., 2018) and in vitro (Javaherian et al., 2015). Here we show that tension not only prevents cell mixing, but also contributes to maintaining the ME boundary cable by stabilizing myosin. Strain and tension regulate myosin dynamics in mitotic Dictyostelium cells (Effler et al., 2006), as well as in the Drosophila embryo during axis elongation (Fernandez-Gonzalez et al., 2009; Scarpa et al., 2018) and wound repair (Kobb et al., 2017; Zulueta-Coarasa and Fernandez-Gonzalez, 2018). Of note, the ME boundary cable is not actively reducing its length, indicating that the regulation of myosin dynamics by tension is not an exclusive feature of actively contracting actomyosin networks.

Strikingly, the myosin cable at the ME boundary prevents premature internalization of the mesectoderm. Epithelial folding is often mediated by an increase in actomyosin activity, either within ingressing cells (Escudero et al., 2007; Martin et al., 2009; Ku and Sun, 2017) or through the assembly of an actomyosin cable at the fold site (Escudero et al., 2007; Ku and Sun, 2017; Urbano et al., 2018). Our data suggest that internalization of the mesectoderm is accompanied by weakening of the actomyosin cables at the ME boundary (Figure 3.3A), suggesting that ME cables may act as “ropes” that maintain the mesectoderm on the surface of the embryo. Ectopic disruption of ME cables may thus result in a collapse of the mesectoderm. In addition, our data reveal that loss of tension at the ME boundary allows ectoderm cells to divide into the ventral midline, thus contributing to the early internalization of the mesectoderm. Furthermore, reduced myosin levels at the ME boundary lead to increased protrusive activity in the ectoderm, which may promote migration of ectoderm cells into the ventral midline. In cell culture systems, Rok inhibition via Y-27632 can upregulate Rac, a Rho GTPase that promotes protrusive activity, resulting in increased cell migration (Tsuij et al., 2002; El-Sibai et al., 2008). Rok also functions upstream of cofilin, an actin-severing protein that can promote protrusive behaviour by increasing the available pool of monomeric actin and the number of barbed ends of actin filaments (reviewed in (Bravo-Cordero et al., 2013)). Cofilin activation, through disruption of Rok, could produce more monomeric actin available for protrusive recruitment (Rac-dependent or otherwise). Indeed, protrusion assembly requires cofilin activity in fibroblasts and metastatic cells, respectively
(Nishita et al., 2004; Yamaguchi et al., 2005), and inhibition of mammalian Rho-kinase in cultured migrating cells increases protrusive activity in a cofilin-dependent manner (Worthylake and Burridge, 2003). Our results suggest that Rok, in addition to regulating myosin-based compartment boundaries, could provide a secondary function in suppressing filopodial assembly and cell motility in order to retain mesectoderm position at the surface of the ventral midline until its internalization during later stages of embryonic development.

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Chapter 4: Discussion

In this chapter, I summarize my primary findings, and I explore how they fit within the current literature. I also consider outstanding questions and future directions inspired by my work.

4.1 Using the Drosophila embryo to study cell mechanics: advantages, limitations, and alternative approaches

Many morphogenetic processes rely on coordinated cell behaviours; however, the mechanisms that drive highly stereotyped tissue movements, and ensure robust organism-to-organism repeatability during development, remain unclear. In the present work, I investigated the role of tensile and molecular signals during axis elongation and ME boundary formation, two morphogenetic processes occurring during development of the early Drosophila embryo. The fruit fly has long been a popular model organism in the scientific community. Over 980 human disease model reports, which link specific human diseases with Drosophila genes, are available on FlyBase today (Gramates et al., 2017). This, alongside the genetic tractability of the fruit fly, makes it an ideal organism to conduct genetic screens in order to identify the molecular mechanisms behind different developmental processes. The fly embryo is amenable to a wide variety of treatments—the transparency of the sac that surrounds the embryo allows for live imaging, and many pharmacological reagents and genetic tools are available and readily accessible. In addition to the short lifespan and straightforward husbandry required of the adult flies themselves, these reasons demonstrate why the Drosophila embryo is ideal for studying in vivo morphogenetic processes that are not yet reproducible in vitro.

With the advent of advanced technologies available for biophysical study of cells and organisms, it has become a reality in recent years to study the role that mechanical forces play in regulating (sub)cellular dynamics. One common way to probe cellular- and tissue-level mechanics is through the use of laser ablation, in which a high-intensity laser is used to
irradiate and sever a subcellular structure. The movements that follow ablation of junctions, actomyosin networks, or microtubule bundles can then be used to estimate the mechanical properties of these structures. Laser ablation has been used to infer relative levels of tension sustained by single junctions (Hutson et al., 2003; Farhadifar et al., 2007; Fernandez-Gonzalez et al., 2009; Yu and Fernandez-Gonzalez, 2016; Chapter 2) and by tissues (Martin et al., 2009; Behrndt et al., 2012; Wang et al., 2017), as well as to apply (Campinho et al., 2013) ectopic tension, and reduce the forces sustained by groups of cells (Wang et al., 2017; West et al., 2017). Still others have used laser ablation to isolate cells from neighbouring tissues (Hutson et al., 2003); in cauterization studies, where the laser presumably fuses tissues to membranes in order to physically constrain cellular movement (Collinet et al., 2015); and to induce wounds in the study of wound repair (Kiehart et al., 2000; Wood et al., 2002; Kobb et al., 2017; Hunter et al., 2018; Zulueta-Coarasa and Fernandez-Gonzalez, 2018).

One major advantage of using laser ablation as a biophysical probe is that it induces instantaneous mechanical force release, thus allowing the inference of tensile forces without the need for physical contact with the specimen under examination. Upon focused high-power laser irradiation on a biological sample, plasma is formed from the concentration of free electrons at the targeted site. This plasma absorbs the energy from the laser and, following irradiation, instantaneously deposits the absorbed energy into the biological sample. For longer laser pulses in the picosecond to nanosecond range, energy is further transferred to the sample via thermal diffusion, which can form cavitation bubbles as a by-product of the spike in temperature. The formation and subsequent implosion of cavitation bubbles then causes targeted damage to the tissue, rendering what is now interpreted as laser ablation (Ma et al., 2009; reviewed in Zulueta-Coarasa and Fernandez-Gonzalez, 2015).

It is important to note that laser ablation has limitations, as irradiation with a high-power laser causes tissue damage. High-power lasers have been reported to induce the release of calcium (Antunes et al., 2013) and reactive oxygen species (Razzell et al., 2013) in vivo. In addition, wound-associated responses are typically observed within 3-5 minutes after wounding, where reparation of the plasma membrane in *Xenopus* oocytes (Davenport et al.,
and cytoskeletal architecture in *Drosophila* embryos (Fernandez-Gonzalez and Zallen, 2013) are among some of the known processes to take place following tissue damage. Although many laser ablation experiments typically span 1-2 minutes, using this technique to study cell and tissue mechanics beyond instantaneous recoil has the potential for damage-associated signals to obscure, or even interfere with, the processes being examined. It would therefore be beneficial to identify alternative, less invasive methods to study cell and tissue mechanics that could replicate some of the findings obtained through laser cutting.

Methods traditionally used to study cell mechanics *in vitro* have recently applied to developing tissues. For example, traction force microscopy (TFM) can be used to infer the forces that cells exert on their substrate by measuring substrate displacement. Recently, a TFM-like method was applied to measure force production *in vivo* during the closure of a dorsal discontinuity in the *Drosophila* embryo through spatial 3D tracking of extracellular matrix (Goodwin et al., 2016). More commonly, TFM is conducted *in vitro* by measuring the displacement of beads embedded in the substrate, such as during tissue extension in *Xenopus* explants (Zhou et al., 2015). This method could then be extended, in addition to monitoring endogenous force patterns, to accommodate for mechanical manipulations—for example, the generation of experimental setups that allow for stretching or compression of tissue isolates has been developed for *in vitro* studies (Foty et al., 1994; Foty et al., 1996; Mgharbel et al., 2009), and combining these techniques with traction force measurements could be fruitful in future studies. Being able to mechanically perturb cells allows for a wider flexibility in studying mechanical properties of tissues, particularly if the experimental setup is amenable to precision in force measurements. Atomic force microscopy (AFM) has proven useful in this respect; in AFM, a sample surface is scanned using a sharp tip attached to a cantilever, and high-resolution topographical images are produced based on cantilever deflection patterns. The ability to fix the cantilever with a known quantity of force further allows measurement of adhesive and mechanical properties; for example, to identify differences in adhesion strength between germ layers during gastrulation in embryonic zebrafish tissues (Krieg et al., 2008) and to pattern stiffness gradients in elongating *Drosophila* egg chambers (Chlasta et al., 2017; Crest et al., 2017). *Drosophila* embryos are encased in a transparent sac, the vitelline membrane, which is necessary for embryogenesis (Munster et al., 2019).
Because the vitelline membrane cannot be removed, the embryonic tissue cannot be directly accessed by the AFM probe. Since the vitelline membrane is likely to interfere with stiffness measurements obtained by AFM, the fly embryo is not ideal for the application of this technique.

Other methods have been adapted to measure and manipulate forces in vivo, and in particular, in fly embryos. Micropipette aspiration has been used to measure cellular responses following application of ectopic tension in many model organisms, including during Dictyostelium (Effler et al., 2006), Drosophila (Fernandez-Gonzalez et al., 2009) and zebrafish (Maitre et al., 2012). To this end, fine-tune calibration methods have been developed to maximize precision of applied forces in microorganism studies in C. elegans and Chlamydomonas reinhardtii (Backholm and Baumchen, 2019). Other techniques have been utilized to directly manipulate cells, including optical (Bambardekar et al., 2015) and magnetic (Desprat et al., 2008; Doubrovinski et al., 2017) tweezers. In optical tweezers, a highly focused laser beam generates a strong electric field, which enables particle trapping. Similarly, in magnetic tweezers, cells are typically incubated with a magnetic ferrofluid, which can be manipulated using magnets external to the sample. Optical and magnetic tweezers have been successful in the controlled application of external forces, and have resulted in novel in vivo measurements of cortical stiffness and cytoplasmic viscosity, two parameters that would be impossible to identify without direct manipulation. Though these techniques have much potential in in vivo studies, they do require specialized equipment and the use of cell impermeable glass/polystyrene or magnetic beads. In Drosophila embryos, beads need to be injected through the vitelline membrane before cells form, and their extended presence in the animal may cause viability problems as development progresses, as foreign fluids, and ferrofluids in particular, can be toxic to embryos (Desprat et al., 2008). Finally, the injection of oil droplets of known mechanical properties into tissues allows inference of force measurements from the deformations sustained by the droplets, and this has been utilized in vitro, in zebrafish embryos, and in mouse mandible explants (Campás et al., 2013; Lucio et al., 2015; Mongera et al., 2018). Similar oil droplet injections have yet to be performed in fly embryos without disrupting normal morphogenesis; one major pitfall is that the deposition of large droplets, as well as large magnetic beads, inside the embryo is
limited by needle size, which must be minimized in order to reduce the degree of epithelial damage.

Vertex models are often used to predict cell shape changes given a set of forces (Hutson et al., 2003; Farhadifar et al., 2007; Zulueta-Coarasa and Fernandez-Gonzalez, 2018). In addition, vertex models can be used to solve the inverse problem and determine the set of forces responsible for a cell shape change. These computational methods minimize the need for technically difficult experimental procedures. For example, many groups have inferred cell-cell stresses from observed strain (Brodland et al., 2010; Sugimura and Ishihara, 2013). The use of computational modelling can further clarify relative force contributions from multiple sources in more complex developmental environments, such as during dorsal closure in *Drosophila* embryos (reviewed in Yu and Fernandez-Gonzalez, 2017; Appendix A). However, though informative, these techniques predict only theoretical values and do not allow for force manipulation.

The advent of improved molecular tools remains a promising source of new methods to measure forces *in vivo*. Molecular force sensors are continuously being developed in response to the aforementioned limitations of various biophysical techniques. In particular, FRET (Förster Resonance Energy Transfer) sensors have secured an important place in the study of cell mechanics (Grashoff et al., 2010; Borghi et al., 2012; Cai et al., 2014; Kelley et al., 2015; Yamashita et al., 2016). FRET technology is based on the energy transfer from one fluorophore to another, whose efficiency is proportional to the distance between the two proteins. However, the use of FRET in fly embryos is limited by a low signal-to-noise ratio (Cost et al., 2015), and this could have played a role in the inability to replicate FRET-based tension measurements using other methods such as laser ablation (Eder et al., 2017). As such, FRET is generally limited to experiments conducted in fixed samples or cultured cells, in systems where background can be minimized. Similarly, quantification of fluorescence of GFP-tagged mechanosensitive proteins has been used as a proxy to measure force dynamics (Kale et al., 2018). As well, the use of optogenetic approaches to (in)activate pathways implicated in regulation of cytoskeletal dynamics is rapidly emerging as an effective method to manipulate mechanical forces *in vivo*, with improved spatial and temporal resolution, and
with reduced physical damage inflicted to cells (Guglielmi et al., 2015; Kaur et al., 2017; Izquierdo et al., 2018; Kong et al., 2019). The promise of improved tools, as they are generated and become more readily available, will undoubtedly make strides in the field of cell mechanics and tissue morphogenesis.

4.2 Factors directing de novo junction formation during axis elongation

In Chapter 2, I established the role of local mechanical signals in the context of axis elongation in the Drosophila embryo. Axis elongation is a conserved process in which the head-to-tail axis of an embryo is established. In Drosophila, axis elongation is primarily driven by cellular rearrangements termed neighbour exchanges, in which AP interfaces contract to form multicellular vertices that resolve through the assembly of new DV junctions. Laser ablation showed that nascent interfaces sustain higher levels of tension than non-elongating ones. I found that new DV interfaces elongate in a pulsatile manner, and that pulses of DV interface elongation correlated with apical area oscillations of the cells immediately anterior and posterior to the resolving vertex. Using pharmacological and biophysical manipulations, I showed that the rate and directionality of new interface assembly requires actomyosin contractility. Finally, using a laser-based technique that I developed to promote apical constriction in cells, I found that increasing tension along the stereotypical axis of interface assembly accelerated junctional elongation, while applying tension in the perpendicular direction re-oriented the new junction parallel to the axis of applied tension. These findings indicate that local, periodic contractile forces polarize junctional assembly to promote global tissue movements during Drosophila axis elongation. However, the molecular mechanisms by which pulling forces from the cells adjacent to a multicellular vertex orient the assembly of the new junction remain unclear.

4.2.1 Tricellular vertices as a mediator of junctional assembly?

Tricellular junctions (points of contact between three cells, TCJ) are sites of major cell rearrangement during cell intercalation. As a multicellular vertex forms and resolves, the TCJs on either side of the contracting junction also exchange cell neighbours during this process (Figure 4.1). Epithelia act as physical barriers to protect the organism from their
Figure 4.1: TCJ rearrangement and cell geometry during neighbour exchange.

During a 4-way neighbour exchange, both TCJs at the ends of the junction that is disassembled will take on a fourth cell neighbour once AP contraction produces a multicellular vertex. The vertex then gives rise to two new TCJs, each replacing one of the original neighbours with a new one.
external environment, and their function requires continuity between cells. This must be satisfied both at interfaces where two cells meet (what is usually referred to as a cell-cell junction), and also at regions where three or more cells meet. Indeed, disrupting TCJ structure results in increased proliferation and impaired barrier function in the fly midgut (Resnik-Docampo et al., 2017), deafness in the mouse due to inability to conduct sound vibrations (Wilcox et al., 2001; Ben-Yosef et al., 2003), and amplified permeability at the mouse blood-brain barrier (Sohet et al., 2015). Recent studies have begun to unravel the complexity of TCJ structure and composition. In vertebrates, TCJs are enriched with the tight junction proteins Tricellulin and Angulin, both of which are required for TCJ sealing (Mariano et al., 2011; Furuse et al., 2014). Tight junctions are analogous to septate junctions in the fly, and the septate junction-associated proteins Gliotactin, Sidekick, and Anakonda are all essential at TCJs in Drosophila (Schulte et al., 2003; Lye et al., 2014; Byri et al., 2015). However, septate junctions are only starting to form in the early Drosophila embryo, and adherens junctions act as the only functional adhesion complexes at this stage. Whether adherens junction complexes are required at TCJs to maintain epithelial continuity is currently unclear. Loss of function experiments for different candidate TCJ components, together with unbiased, image-based genome-wide screens, will reveal the factors necessary to maintain TCJ sealing in the early Drosophila embryo.

Furthermore, super-resolution imaging techniques are increasingly being applied to living samples, further allowing to probe the architecture of TCJs in living embryos undergoing cell rearrangements. One such technique is super-resolution radial fluctuation microscopy (SRRF), which utilizes a series of rapidly-acquired fluorescence image sequence to reconstruct an image of the sample with a spatial resolution as low as 60 nm, without the need for specialized microscope setups (Gustafsson et al., 2016). As opposed to other super-resolution techniques such as PALM or STORM, which entails image acquisition of fixed samples over long periods of time, SRRF reconstructions can be generated with a temporal resolution of as little as 1 second using Graphics Processing Units (Gustafsson et al., 2016). This makes it an ideal method for increasing spatial image resolution of live samples without compromising temporal resolution nor modifying existing microscopy setups. As well, electron microscopy of the intercalating ectoderm would confirm whether the epithelium is
continuous at TCJs, or if transient gaps emerge as a result of dynamic cell movements. These experiments would also be vital to establish the structure of multicellular vertices during intercalation: due to their transient nature, discontinuities could arise as multicellular vertices form. Indeed, myosin forms rings around multicellular vertices as they form (Sawyer et al., 2011), not unlike the actomyosin structures that close wounds in the Drosophila epidermis (Wood et al., 2002). Myosin rings around rosette vertices could help to reinforce epithelial sealing until the vertex resolves. Quantitative imaging at a high temporal and/or spatial resolution, alongside injection of cell-impermeable dyes, would help to reveal how multicellular vertices form and dissociate without affecting overall epithelial integrity, and provide clues as to why multicellular vertices, in comparison to TCJs, are much more structurally unstable.

Enrichment of adherens junction components at TCJs raises an interesting possibility when we consider junctional association with the cytoskeleton. E-cadherin is present as a transmembrane protein, forming cis- and trans- connections with neighbouring E-cadherin molecules to induce clustering (Harris and Tepass, 2010; Wu and Yap, 2013). Meanwhile, β-catenin binds to the cytoplasmic tail of E-cadherin (Huber and Weis, 2001) and can recruit α-catenin (Pokutta and Weis, 2000), which can bind directly to actin filaments, particularly under cytoskeletal tension (Buckley et al., 2014). During Drosophila wound closure, E-cadherin, α-catenin, and β-catenin become enriched at TCJs at the wound edge and, correspondingly, becomes depleted at interfaces between wounded and unwounded cells, while actin and myosin accumulate at the wound margin (Wood et al., 2002; Abreu-Blanco et al., 2012; Zulueta-Coarasa et al., 2014; Hunter et al., 2015). Thus, adherens junctions at TCJs may act as anchor points for the wound cable, and further to this, it has been proposed that actin polymerization radiates outwards from anchoring TCJs (Hunter et al., 2015; Matsubayashi et al., 2015). Accordingly, in cell culture, myosin VI acts as a force sensor and triggers mDia1-mediated actin polymerization at junctions while reinforcing the integrity of adherens junctions at multicellular vertices (Acharya et al., 2018). Additionally, cells in the mouse limb bud ectoderm display pulsed contractions that are disrupted in β-catenin mutants, and in these mutants the directionality of vertex resolution is lost (Lau et al., 2015). Locally disrupting adherens junctions at TCJs associated with nascent interfaces (e.g. by using
optogenetic tools), and tracking actin polymerization at the new interface, will shed light on whether adherens-junction-assembly at TCJ is vital for actin polymerization and assembly of new junctions.

The signals that promote actin polymerization at new junctions are currently unknown. Actin is enriched at multicellular vertices, and during vertex resolution, E-cadherin and actin localize to new junctions before other proteins do (Blankenship et al., 2006). Careful measurements correlating actin fluorescence at the new junction with pulsatile behaviours in neighbouring cells could help to identify patterns of actin assembly at elongating interfaces. Preliminary data suggest that actin localizes to vertices before junctions (Appendix B, Figure B1), which indicates that actin polymerization could originate from vertices in the germband. Experiments employing Fluorescence Recovery After Photobleaching (FRAP) on TCJs could reveal actin polymerization dynamics during the formation of new ectodermal junctions. If actin radiates outwards from TCJs, then photobleaching TCJs at new junctions should result in photobleached actin moving into the junction, leading to overall decreased fluorescence levels compared to controls. In contrast, if junctional fluorescence did not decrease, this could be indicative of actin polymerization originating at the junction itself. Alternatively, actin polymerization could be occurring without positional preference; if this is true, then tracking line profiles of bleached interfaces over time should result in homogenous fluorescence recovery along the entire junction. Either outcome could indicate that the cell has mechanisms to direct actin polymerization at sites of cortical discontinuity, particularly as vertices begin resolving into new DV interfaces. Using an optogenetic approach to locally disrupt the actin cortex (Guglielmi et al., 2015) on sub-interfacial scales, combined with XZ timelapse imaging, could help to uncover how actin polymerization and recovery can mend subcellular cortical discontinuities. These experiments will help to elucidate the contribution of actin polymerization at different interfacial domains towards the formation of new junctions during tissue extension.

4.2.2 Mechanical feedback during cell intercalation

Tissue extension in the fruit fly embryo is a highly dynamic process, with several sources of tensile stress of varying spatial scales (Collinet et al., 2015; Lye et al., 2015; Yu and
Fernandez-Gonzalez, 2016; Chapter 2). Both local and global sources of tension are necessary for proper tissue extension. In the germband, myosin localizes to both the junctional domain at AP interfaces, facilitating their contraction (Bertet et al., 2004; Zallen and Wieschaus, 2004; Rauzi et al., 2008; Fernandez-Gonzalez et al., 2009), as well as the apical-medial domain, driving oscillatory cell contractions (Fernandez-Gonzalez and Zallen, 2011; Levayer et al., 2011; Sawyer et al., 2011). Previous work from us and others indicates that the pulsatile contractions of the apical cell surface promote vertex resolution (Collinet et al., 2015; Yu and Fernandez-Gonzalez, 2016; Chapter 2). In addition, the contribution of myosin to elongate nascent germband interfaces may result in part from its absence from those interfaces. In the Drosophila pupal wing, junctions lengthen only when myosin leaves the new interface, as a result of the activity of the tumour suppressor PTEN; when PTEN is downregulated, myosin localization at new interfaces is sustained, and new junctions undergo cycles of shortening and lengthening (Bardet et al., 2013). Indeed, when the planar polarity of myosin is lost in the germband, intercalation occurs without directional bias and the tissue does not extend (Irvine and Wieschaus, 1994; Zallen and Wieschaus, 2004; Blankenship et al., 2006). It is possible that the reduction of myosin alters the mechanical properties of the nascent junction itself; a reduction in junctional tension could enhance interfacial plasticity/elasticity, which could be conducive to junctional assembly and/or stabilization as neighbouring cells pull on the junction. Further experiments that allow for the local manipulation of myosin activity, for example, in flies where myosin phosphorylation can be controlled spatiotemporally by optical light (in)activation, could help to clarify these ideas further.

My data showing that new junctions exhibit higher tensile forces than pre-existing junctions of the same orientation (Yu and Fernandez-Gonzalez, 2016; Chapter 2) suggest that nascent junctions may require stable anchoring points as they undergo pulsatile, productive elongation. This is especially true if we consider that myosin activity is reduced at new junctions, and that contractile forces originating anterior and posterior to the nascent interface, both locally and globally, facilitate its extension. The two TCJs associated with the new junction could be acting in this role. TCJs have been proposed to act as anchor points within the actomyosin cable that forms around embryonic wounds—as the cable matures,
adhesion proteins are removed from the junctional domain and are recruited to TCJs (Wood et al., 2002; Abreu-Blanco et al., 2012; Zulueta-Coarasa et al., 2014; Hunter et al., 2015). This could potentially act to facilitate actomyosin recruitment and contractility at the interfacial domain between vertices. TCJs also help to polarize pulling forces during cell division in the Drosophila pupal notum, in which TCJs are redistributed with respect to global tissue stresses prior to mitosis, and recruit microtubule-based motors to orient cell division (Bosveld et al., 2016).

The physical structure of germband TCJs has not been studied, but we can speculate as to which players could be active at TCJs. Monomeric α-catenin interacts simultaneously with both actin and the cadherin-β-catenin complex in Drosophila tissues (Desai et al., 2013), and in vitro studies later confirmed that this interaction occurs under mechanical stress (Buckley et al., 2014). Under tensile stress, α-catenin undergoes conformational changes and unfurls to reveal a binding domain that recruits vinculin (Yonemura et al., 2010). Vinculin has also been reported to be mechanosensitive, binding directly to F-actin and promoting the activity of other actin regulators under tensile stress (le Duc et al., 2010; Huveneers et al., 2012). To this end, recent studies have used fluorescent vinculin as a readout for mechanical load, and shown that vinculin recruitment to germband interfaces requires both α-catenin and myosin activity (Kale et al., 2018). Though not formally discussed, fluorescent images indicate that vinculin is enriched at TCJs (Kale et al., 2018), indicating that TCJs bear some degree of stress during cell intercalation. Closer examination of fluctuating vinculin levels at the TCJs flanking elongating interfaces relative to neighbouring cell oscillations, will help to reveal the patterns of mechanical load at TCJs during junctional assembly.

Perhaps the most appealing possibility, when considering players that could be recruited by mechanical forces, is the idea that tension could feed back into itself through cytoskeletal proteins and interactors. Myosin can both promote and be recruited by increased tension (Effler et al., 2006; Fernandez-Gonzalez et al., 2009; Kobb et al., 2017). In addition, the septin cytoskeleton provides support to the actin cytoskeleton by linking it to the plasma membrane (Kinoshita et al., 2002; Joo et al., 2007). In Xenopus, septins are required for myosin planar polarity during convergence and extension movements, and anisotropic
interfacial tension is abolished upon septin inhibition (Shindo and Wallingford, 2014). Additionally, septins and actin are both enriched at TCJs, and actin no longer localizes to TCJs upon septin reduction (Shindo and Wallingford, 2014). This, combined with in vitro studies which suggest that septins could be mechanosensors, both in the recruitment of focal adhesions as well as in curvature sensing (Dolat et al., 2014; Calvo et al., 2015; Bridges et al., 2016), could hint towards a role for septins during Drosophila axis elongation. Further loss-of-function studies will reveal the contribution of the septin cytoskeleton in supporting tissue extension.

Of particular interest during junctional assembly is filamentous actin. Actin is the first known molecule to localize to nascent junctions, presumably to construct a scaffold from which further extension can build upon (Blankenship et al., 2006). Could actin itself be mechanically regulated at these sites? Indeed, mechanical tension can promote actin polymerization in in vitro studies (Courtemanche et al., 2013; Higashida et al., 2013; Jegou et al., 2013), but determining the relationship between tension and actin polymerization during axis elongation requires careful local manipulations in order to reduce crosstalk between AP interface contraction and DV interface elongation, both of which occur simultaneously in the same epithelium. Preliminary data in which ectopic tension was applied to nascent DV interfaces in embryos expressing an actin reporter indicates that actin fluorescence was 2.3-fold greater under applied tension (Appendix B, Figure B2, $P = 0.01$). These data suggest that the pulsatile contractions of cells adjacent to elongating DV interfaces in the germband could promote actin polymerization in a mechanosensitive manner. Further experiments quantifying changes in actin fluorescence in elongating edges, in response to ectopic forces or anterior and posterior contractions, will help elucidate the role of tension in promoting actin polymerization and new interface assembly during Drosophila axis elongation.

Other actin regulators could be candidates of interest. In particular, proteins that sever or crosslink actin filaments can play critical roles in cytoskeletal remodelling. Cofilin, an actin-severing protein, promotes junctional stability during gastrulation; adherens junctions rupture when Cofilin (and actin turnover) is downregulated (Jodoin et al., 2015). Additionally, in the
Drosophila pupal wing, the Cofilin cofactor AIP1 enhances F-actin turnover at shortening junctions, which is required to balance mechanical load during intercalation events (Ikawa and Sugimura, 2018). Importantly, both Cofilin and AIP1 can be regulated by mechanical forces—Cofilin activity can be mechanically stimulated in cardiac fibroblasts (Zhao et al., 2007), and AIP1 preferentially localizes to interfaces parallel to the axis of tissue stretch in the Drosophila pupal wing epithelium (Ikawa and Sugimura, 2018). Sites of increased tension can also recruit other actin crosslinkers, such as \(\alpha\)-actinin (through \(\alpha\)-catenin) (Nieset et al., 1997), filamin (Glogauer et al., 1998; Furuike et al., 2001; Huelsmann et al., 2016), and myosin (Effler et al., 2006; Fernandez-Gonzalez et al., 2009; Kobb et al., 2017). Cytoskeletal remodelling can occur as a consequence of actin filament disassembly, when newly-severed filaments reattach via crosslinking proteins to maintain cortical integrity (Zumdieck et al., 2007). Therefore, actin disassembly could be helping to stabilize new cytoskeletal scaffolds as new actin filaments are being polymerized, resulting in processive interfacial elongation. FRAP experiments could help to elucidate facets of actin turnover and confirm to what degree actin (dis)assembly occurs at nascent junctions, and further investigation using local silencing of actin severing/crosslinking proteins will reveal how actin dynamically builds new DV interfaces during axis elongation.

4.2.3 Molecular regulators of actin structures at nascent interfaces
Filamentous actin is the first protein to localize to the new junction (Blankenship et al., 2006), and my preliminary data suggest that pulses of actin assembly promote pulses of interfacial elongation during vertex resolution (Figure 4.2). Furthermore, new DV junctions fail to assemble when actin polymerization is inhibited (Yu and Fernandez-Gonzalez, 2016; Chapter 2), indicating that actin polymerization is essential for junctional assembly. Despite this, the signals that recruit actin to these sites, and the factors driving its polymerization, remain unknown. One way to determine which actin regulators are active in intercalating cells is to conduct a candidate RNAi screen for actin regulators necessary for germband extension. In preliminary work, and I used this approach to screen 18 candidates (Appendix C, Table C1). In this section, I will briefly present some of my preliminary data, discuss my findings and shortcomings, and outline routes for further study.
As discussed in the previous section, it is possible that pulsed tensile stresses could be promoting actin polymerization directly. The formin family of proteins adds actin monomers to the plus end of single, unbranched filaments, and in \textit{in vitro} systems, can increase throughput in the presence of increased tension, making them ideal candidates for further study (Courtemanche et al., 2013; Higashida et al., 2013; Jegou et al., 2013). There are six formins in \textit{Drosophila}: Cappuccino (Capu), Dishevelled Associated Activator of Morphogenesis (DAAM), Formin homology 2 domain containing ortholog (Fhos), Formin-like (Frl), Formin3 (Form3), and Diaphanous (Dia). Using RNAi lines from the Harvard Transgenic RNAi Project (TRiP), I found that only Dia knockdown results in germband extension defects (Appendix C, Figure C1). Dia has been implicated in the contraction of AP interfaces during \textit{Drosophila} neighbour exchange (Levayer et al., 2011), but its role during and following vertex resolution is still unknown. Follow-up study with higher magnification to track deficits in both contraction and elongation rates in loss-of-function flies will be necessary to fully establish the role of Dia in different junctional domains during cell intercalation.

Cofilin is necessary to establish planar cell polarity (Blair et al., 2006) and for endocytosis to proceed (Lappalainen and Drubin, 1997), and both cell polarity and endocytosis are critical for germband extension. Consistent with this, downregulation of Slingshot (Ssh), a phosphatase that activates Cofilin (Niwa et al., 2002), resulted in germband extension defects (Appendix C, Figure C2A-C). At the cellular level, germband cells displayed abnormal shapes and sizes, and the tissue eventually tore (Appendix C, Figure C2D-E), which is in line with previous studies that establish a role for Cofilin during mesoderm invagination (Jodoin et al., 2015). Similarly, I observed whole-embryo defects occurring before the onset of germband extension upon knockdown of Arpc4, a component of the Arp2/3 complex which nucleates branched actin structures; Ciboulot and Chickadee (the \textit{Drosophila} homolog of profilin), which both bind to actin monomers; and Quail, an actin bundling regulator. Knockdown of each by RNAi resulted in embryos that were too fragile to be handled for microscopy, which indicates that the activity of each of these candidates is required during
earlier stages of development. Thus, a method to temporally regulate the degree of knockdown during embryonic development is necessary to bypass earlier essential functions.

Germband extension occurs just before the onset of zygotic transcription, and until this point, the embryo depends on materials contributed by the mother; thus, the method of maternal RNAi expression used in this initial screen is limited to identifying candidates that are not essential for earlier stages of embryogenesis. This is problematic when we consider that protein functions could be redundant across developmental processes. One way to circumvent this issue is through the injection of pharmacological agents. Thus, a drug screen utilizing commercially available drug libraries could be a viable alternative to RNAi, validating the top hits using secondary genetic approaches to eliminate off-target effects by the drugs. Further validation of screen hits could then benefit from the generation of new fly lines to generate GFP-tagged proteins of interest and/or flies with mutations at certain loci, for example using CRISPR-Cas9 technology (Bassett et al., 2013), and studying intercalary events to determine whether vertex resolution is affected. Together, these approaches could produce an improved, more efficient method for screening through actin regulators that are required for germband extension.

4.3 Mechanisms underlying tissue boundary formation and maintenance during embryonic fly development

In Chapter 3, I used the early Drosophila embryo as a model to study the formation and maintenance of tissue boundaries. A polarity system results in the formation of supracellular myosin cables which segregate the mesectoderm from the ectoderm. Tissue compartments are maintained through increased cable tension at the boundary, accompanied by stabilization of myosin at the tissue barrier, and myosin is reduced at the boundary upon loss of cable tension. Boundaries disintegrate upon complete inhibition of myosin-based contractility, resulting in ectodermal invasion and premature mesectodermal internalization. These findings demonstrate how myosin cables maintain tissue segregation and position during the development of the early Drosophila embryo.
4.3.1 Organization of molecular polarity during tissue boundary formation

My data show that the ME boundary arises through a reversal of planar polarity in the mesectoderm, established following the completion of cell division. As mesectoderm daughter cells re-establish their cortical composition, Bazooka, Rok, and myosin become planar polarized to complementary domains (Bazooka to AP junctions, Rok and myosin to DV junctions; Chapter 3, Figure 3.1). This is reminiscent of similar molecular patterns occurring earlier in the ectoderm during germband extension, where planar polarity of adhesion and cytoskeletal components emerges to direct global convergence and extension movements. During this process, Bazooka localizes to DV junctions, while Rok and myosin preferentially localize to AP junctions (Bertet et al., 2004; Zallen and Wieschaus, 2004; Blankenship et al., 2006; Simoes et al., 2010). In both the ectoderm and the mesectoderm, Bazooka and myosin polarity is abolished upon knockdown of Rok (Bertet et al., 2004; Fernandez-Gonzalez et al., 2009; Simoes et al., 2010). In the ectoderm, Rok activity is modulated by the cytoskeletal regulator Shroom, which is in turn controlled by Rho GTPase (Simoes et al., 2014). Rho activity depends on RhoGEF2 to promote downstream contractility (Levayer et al., 2011; Kerridge et al., 2016), as well as Smog, a G-coupled protein receptor (Kerridge et al., 2016). Further studies to determine the involvement of Smog, RhoGEF2 and Rok in the polarity system of the mesectoderm will clarify whether these proteins play similar roles in both tissues.

Planar polarity in the ectoderm arises from embryonic patterning genes (Irvine and Wieschaus, 1994). Thus, differential patterning cues or cell fate determinants could be responsible for our observed reversal of polarity in the mesectoderm. Mesectoderm cells express the transcription factor Single-minded (Sim) (Springer and Rutschky, 1969; Nambu et al., 1991), and mesectoderm cells are missing in sim mutants (Nambu et al., 1991), instead becoming integrated with the ectoderm or undergoing apoptosis (Xiao et al., 1996). Sim expression requires the transcription factor Twist—while mesoderm cells express Twist and the transcriptional repressor Snail, mesectoderm cells express only Twist, and ectoderm cells express neither (Kosman et al., 1991; Rao et al., 1991). Thus, Twist and Snail are expressed in two rectangular stripes in the ventral surface of the embryo, with a common centre, but a greater width for the Twist stripe. The parallel boundaries of Twist and Snail are controlled
by Dorsal, a patterning gene responsible for dorsal-ventral axis specification (Morisato and Anderson, 1995). Interestingly, Sim expression also relies on maternally-contributed Notch (Morel and Schweisguth, 2000), a receptor family that has been implicated in establishing boundaries in other systems, including the chick brain, the mouse cochlea, and the fly wing disc (Tossell et al., 2011; Basch et al., 2016; Michel et al., 2016). Misexpression of mesectoderm fate determinants in the early _Drosophila_ embryo could reveal the extent to which differential cell polarities are regulated by mesectoderm fate. Ectopic, heat shock-induced expression of Sim in the lateral ectoderm can induce mesectodermal identity (Nambu et al., 1991). In this scenario, it would be interesting to test if actomyosin boundaries form between ectopically-directed mesectoderm and ectoderm, which would suggest that cells can recognize like and unlike cells independently of position on the embryonic body, and that boundaries arise downstream of differential cell fate specification.

Since differential polarities do not arise until after mesectoderm cells divide, it is possible that mesectoderm repolarization originates from mechanisms associated with mesectoderm cell division. I proposed in Section 3.5 that Bazooka could be polarized to AP junctions in the mesectoderm as a consequence of the bias in division angle towards the long axis of the embryo (Wang et al., 2017), since Bazooka has been implicated in the orientation of cell division (Mauser and Prehoda, 2012). Bazooka is depleted at myosin-enriched boundaries in other tissues (Wei et al., 2005; Major and Irvine, 2006; Urbano et al., 2018). If the assembly of new interfaces between daughter mesectoderm cells recruit Bazooka to AP junctions and is consequently sequestered away from DV junctions, myosin could be redistributed and recruited to the ME interface as a result. This assumes that the mechanisms to retain complementary polarity between Bazooka and myosin, as observed in the ectoderm (Simoes et al., 2010), are similar in the mesectoderm. Studying fly embryos in which cell division is inhibited—for example, using flies with mutations in _string_, a Cdc25-type phosphatase that activates the mitotic kinase Cdk1 and is required for post-blastoderm cell division (Edgar et al., 1994)—could help to clarify whether cell division is important for mesectoderm cells to repolarize during the establishment of the ME boundary.
4.3.2 Maintenance of ME boundaries until tissue internalization

My data suggest that ME boundaries require myosin and tension for long-term segregation of ectoderm from mesectoderm. Although I have demonstrated this using a biophysical perturbation (Chapter 3, Figure 3.3) and a pharmacological treatment (Chapter 3, Figures 3.4 and 3.5), further experiments using genetic manipulations will confirm these findings. As discussed in Section 4.1, laser-based techniques induce tissue damage, which could trigger downstream effects that could interfere with the behaviour of cells. As well, pharmacological treatments can have off-target effects; Y-27632 in particular is known to affect the atypical protein kinase C (aPKC) (Atwood and Prehoda, 2009). aPKC phosphorylates Bazooka, and this confines Bazooka to adherens junctions, which contributes towards junctional stability (Krahn et al., 2010; Morais-de-Sa et al., 2010; Walther and Pichaud, 2010). Thus, indirectly targeting aPKC with Y-27632 could affect junctional integrity. Genetic perturbations specific to myosin activity would help to reduce off-target effects and clarify effects on boundary formation and/or maintenance—for example, through the generation of sqh germline clones (Karess et al., 1991), the expression of phosphomutant myosin on a null background (Vasquez et al., 2014), or the overexpression of a dominant negative form of myosin (Dawes-Hoang et al., 2005).

Boundary formation between ectoderm and mesectoderm occurs within 20 min, during which junctions undergo extensive remodelling. However, once the ME boundary is fully established, it remains in place as a barrier between the two tissues until the retraction of the germband occurs, approximately 5-6 hours later (Jacobs and Goodman, 1989; Klambt et al., 1991; Tepass and Hartenstein, 1994). My data indicate that the ME boundary loses myosin as the mesectoderm is internalized (Chapter 3, Figure 3.5). Thus, in addition to acting in tissue segregation, tension could regulate the internalization of the mesectoderm. A formal characterization of ME boundaries during wild-type mesectoderm internalization will shed light on how mesectoderm cells ingress, and reveal whether other known mechanisms of cell/tissue internalization also recur here. Before mesectoderm cells divide, the ventral midline forms following the invagination of the mesoderm (Chapter 1, Figure 1.3A). During this process, each mesoderm precursor forms actomyosin networks on its medioapical cortex, facilitating pulsed contractions that processively result in the coordinated constriction of
mesodermal cell surfaces and eventually resulting in tissue folding (Martin et al., 2009). In contrast, neuroblasts ingress from the neuroectoderm epithelium through a completely different mechanism—the polarized localization of myosin first facilitates contraction of AP edges, followed by the formation of new adherens junctions between the cells dorsal and ventral to each ingressing neuroblast (Simoes et al., 2017). Careful analysis of wild-type mesectoderm internalization could identify the presence of medial myosin networks, asymmetric junctional remodelling, or both, which could parallel features of mesoderm invagination or neuroblast ingression; alternatively, it could also reveal novel mechanisms of tissue internalization.

The position of the mesectoderm could be affected by neighbouring tissues, alongside their associated movements throughout development; for three-dimensional scanning electron micrographs of the mesectoderm, refer to (Turner and Mahowald, 1977). For example, it is possible that as the mesoderm migrates inside the body cavity, it exerts a pulling force on the mesectoderm to facilitate its internalization. Characterization of mesectoderm and boundary morphology in snail mutants, in which the mesoderm does not invaginate and instead remains on the surface of the embryo (Martin et al., 2009), would establish whether the mesoderm is necessary for mesectoderm internalization. Another tissue that could be affecting the position of the mesectoderm is the ectoderm, which concludes tissue extension as ME boundaries begin to form. Ectoderm extension is facilitated by a combination of the local directional forces generated within germband cells and the global forces supplied by the internalization of the posterior midgut (Collinet et al., 2015; Lye et al., 2015; Yu and Fernandez-Gonzalez, 2016; Chapter 2). Thus, genetically inhibiting AP patterning, which is necessary for cell intercalation (Zallen and Wieschaus, 2004; Blankenship et al., 2006; Fernandez-Gonzalez et al., 2009), or disrupting the invagination of the posterior midgut could help to uncover whether the tension sustained by ME cables depends on directional forces generated by neighbouring tissues, and elucidate any effects that those tissues may have on myosin recruitment/stability.

The maintenance of the ME boundary is in contrast with other supracellular actomyosin cables that are disassembled as they contract (Kiehart et al., 2000; Fernandez-Gonzalez et al.,
2009; Lienkamp et al., 2012; Nishimura et al., 2012; Kobb et al., 2017). Although the ME boundary is enriched with myosin and is under tension, it does not contract, and the reasons for this remain unclear. I speculate that the force balance resulting from the linear (not elliptical nor circular) geometry of the cable, and the distribution of myosin in the cable, are responsible for the maintenance of the ME boundary (Figure 4.2A). A heterogeneous distribution of myosin has been reported in the contractile cables that promote rosette formation during germband extension (Fernandez-Gonzalez et al., 2009). Importantly, a similar heterogeneous distribution of actin and myosin facilitates the contraction of individual segments of the cable around embryonic wounds, and when a homogenous distribution of myosin was computationally simulated, wound closure rates diminished (Zulueta-Coarasa and Fernandez-Gonzalez, 2018). Thus, I hypothesize that myosin levels are uniform along the ME boundary, a prediction that can be easily tested. A preliminary visual assessment suggests that myosin appears more uniform at the ME boundary than around embryonic wounds (Figure 4.2B-C). Heterogeneity at the wound margin is at least in part mediated by Abl, a tyrosine kinase that localizes to the wound edge and regulates actin localization and dynamics (Zulueta-Coarasa et al., 2014). I further predict, as ME boundaries do not appear to exhibit a large degree of myosin heterogeneity, that Abl will not localize to the ME boundary, and that the ME boundary will persist normally in abl mutants (assuming that Abl is not required for the initial assembly of the ME boundary).

4.3.3 Is differential adhesion a feature of the ME boundary?

Our data implicating a role for actomyosin contractility in maintaining compartment boundaries at the ME interface is in line with other studies in flies (Landsberg et al., 2009; Monier et al., 2010; Aliee et al., 2012; Umetsu et al., 2014a; Scarpa et al., 2018; Urbano et al., 2018), zebrafish (Krieg et al., 2008; Calzolari et al., 2014), frogs (Rohani et al., 2011; Fagotto et al., 2013; Rohani et al., 2014), and mice (O'Neill et al., 2016). Moreover, our data reveal that tension may help stabilize myosin at boundaries, reminiscent of myosin cables in the Drosophila ectoderm (Fernandez-Gonzalez et al., 2009; Scarpa et al., 2018), and at the wound margin in the Drosophila epidermis (Kobb et al., 2017). Increased tension at heterotypic contacts has been proposed as a means by which tissue barriers can persist (Brodland, 2002; Brodland et al., 2009); however, it is possible that this mechanism is not
Figure 4.2: Homogenous myosin levels under a force balance at the ME boundary may contribute to its maintenance.

(A) Schematics showing cells with myosin planar polarized to ME boundaries (left). Grey box outlines a vertex where interfaces $i_1$, $i_2$, and $i_3$ converge. Force balance of the outlined vertex (right) illustrate the equilibrium state of vectors $T_{1^*}$, $T_{2^*}$, and $T_{3^*}$, representing the tensile forces along $i_1$, $i_2$, and $i_3$, respectively. The thickness of the green arrows reflects relative levels of tension inferred from interfacial myosin enrichment. (B) ME boundaries (marked by yellow dotted lines) in an embryo expressing myosin:GFP. Anterior, left. (C) A myosin cable around a wound in a stage 14 embryo expressing myosin:GFP. Arrowheads indicate regions of high (red) or low (cyan) myosin fluorescence. Image captured approximately 10 min after wounding. Anterior left, dorsal up. (B-C) Scale bars, 10 µm.
acting alone. Differential adhesion can facilitate cell sorting via differences in adhesive strengths (Steinberg, 1963). Indeed, actomyosin contractility and cell-cell adhesion are often intricately linked at compartment boundaries. In particular, the receptor-ligand pair Eph/Ephrin is commonly associated with the maintenance of boundaries, directing actomyosin accumulation (Cortina et al., 2007; Calzolari et al., 2014) as well as downregulation of adherens junctions (Nunan et al., 2015) at tissue borders. Likewise, increased tension as well as differential adhesion are both necessary to maintain compartment boundaries in Drosophila wing disc clones and between zebrafish germ layers, respectively (Chang et al., 2011; Maitre et al., 2012). In the context of the ME boundary, a role for differential adhesion remains unexplored.

Adhesion, mediated by the association of junctional complexes between cells, is often downregulated at tissue boundaries. For example, at the Xenopus notochord-paraxial mesoderm boundary, cadherin complexes fail to cluster together and do not form bonds between cells; however, junctional complexes are permitted to associate with each other when actomyosin-based contraction is inhibited (Fagotto et al., 2013). Additionally, when Echinoid mutant clones are induced in the Drosophila wing disc, junctional downregulation at clonal boundaries accompanies actomyosin accumulation and repolarizes Bazooka towards non-boundary junctions that are cadherin-enriched (Wei et al., 2005). Thus, contractility along the boundary may prevent adhesion complexes from forming/stabilizing across the boundary (Fagotto, 2015). Preliminary data suggests that similar dynamics could be acting at the ME boundary in flies—in addition to myosin enrichment and increased tension (Chapter 3), E-cadherin appears downregulated at the ME interface compared to homotypic junctions within compartments (Figure 4.3). Further experiments would be needed to characterize adhesive properties across the ME boundary. Differences in adhesive strength originate from dissimilarities in junctional composition between cell types, either from the variety or amount (or both) of junctional molecules on either side of the boundary. In the early Drosophila embryo, adherens junctions are the only adhesive complexes present that connect cells; septate junctions (equivalent to vertebrate tight junctions) are not functionally mature until the final stages of embryogenesis (Tepass and Hartenstein, 1994). If
Figure 4.3: E-cadherin levels are reduced at the ME boundary.

ME boundaries in an embryo expressing E-cadherin:GFP (top, green; middle, greyscale) and myosin:mCherry (top, magenta; bottom, greyscale). Yellow dotted lines mark ME boundaries where E-cadherin is depleted. Anterior, left. Scale bar, 10 µm.
E-cadherin is the main junctional cell-cell adhesion protein present in the early embryo, then differences in combinations of cadherins cannot contribute to differential adhesion at the ME boundary; thus, if differential adhesion is present at the ME boundary, it may be due to differences in E-cadherin expression across heterotypic cells.

The Eph/ephrin receptor-ligand pair is commonly associated with compartment boundaries in many systems, with established roles in regulating both adhesion and contractility (Cayuso et al., 2015). Although the link between Eph/ephrin and actomyosin contractility remains unclear, it has been suggested that ROCK-mediated boundary formation is required for unidirectional signalling via Eph, and not ephrin, in the mouse neuroepithelium (O’Neill et al., 2016). Indeed, an RNAi screen to identify genes involved in maintaining the linearity of the anterior-posterior compartment boundary in the Drosophila wing disc implicated the Eph receptor, and the boundary was disrupted only in regions where eph mutant clones present on both sides of the boundary made contact (Umetsu et al., 2014b). Moreover, ephrin overexpression specifically at the mesectoderm results in defects in the ventral nerve cord later in development (Bossing and Brand, 2002). Together, these data suggest that Eph/ephrin could be important for development of the mesectoderm, potentially by regulating the boundaries separating it from the ectoderm. Unlike vertebrates, there is only one Eph receptor and one ephrin ligand in Drosophila, which minimizes the complexity associated with characterizing combinatorial effects of multiple isoforms. Thus, if Eph/ephrin are involved with ME boundary maintenance, differential expression of both should be observed between mesectoderm and ectoderm. To study Eph localization, standard microscopy could be used to image GFP-tagged Eph flies (Nagarkar-Jaiswal et al., 2015), and verify whether Eph is enriched at the ME boundary, while ME boundary formation could be investigated in eph mutants or when ephrin is overexpressed. To this end, I performed live imaging on embryos where ephrin is overexpressed, and my preliminary data indicate that mesectoderm clusters remain stationary under the epidermis following internalization, instead of migrating away from the midline—similar to phenotypes obtained in flies with sim-GAL4-driven Rok or myosin inhibition (data not shown). Another possibility is to study ectoderm-mesectoderm interactions in vitro. One previous study successfully isolated mesectoderm from embryos and cultured them into mature neurons (Luer and Technau,
Using this method, one could isolate ectoderm and mesectoderm cells from wild-type, Eph-overexpressing, and ephrin-overexpressing embryos, and culture cells in vitro together in different genotypic combinations. These experiments will determine if cell sorting can still occur in vitro, and if boundary defects arise with different combinations of Eph/ephrin levels, which would clarify whether differential Eph/ephrin signalling is important for mesectoderm-ectoderm tissue segregation.

4.4 Conclusions
The mechanisms by which cells coordinate their movements during development affect the form and function of mature tissues. Polarized cytoskeletal dynamics contribute to the coordination of cell behaviours, and misregulated cell coordination can lead to cancer metastasis (Gaggioli et al., 2007; Hidalgo-Carcedo et al., 2011). In this work, I have identified mechanisms by which cytoskeletal dynamics can enable or restrict local cell movements during embryonic development. I found that polarized contractile stresses are both necessary and sufficient to orient the assembly of nascent cell-cell junctions during tissue elongation. Additionally, I showed that sustained cytoskeletal tension between compartments is required to maintain tissue segregation. My findings detail how local mechanical cues affect cellular interactions, and provide insight into how subcellular dynamics orchestrate tissue-level movements and coordination during embryonic development.
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Appendix A: Quantitative modelling of epithelial morphogenesis: integrating cell mechanics and molecular dynamics

A modified version of this chapter appeared as:

Appendix A: Quantitative modelling of epithelial morphogenesis: integrating cell mechanics and molecular dynamics

A.1 Abstract
Epithelial tissues form and repair in complex processes influenced by molecular and physical factors. Recent years have witnessed the development of new microscopy modalities that push the limits of spatial resolution, and enable long-term monitoring of developing animals. Increasingly, methods from the physical sciences are used to investigate the role of mechanical forces in living organisms. The application of these new technologies to developmental biology has led to ever-expanding volumes of data that must be interpreted and integrated. For these reasons, computer models are being applied to investigate tissue morphogenesis. Here, we discuss the use of vertex models to study the morphogenesis of epithelial tissues. We motivate the use of computational models and consider their advantages and limitations. We provide an introduction to the theoretical foundation of vertex models and describe how they can integrate mechanical and biochemical dynamics. Finally, we review recent advances in the application of vertex models to investigate dorsal closure, a morphogenetic process in the Drosophila embryo with parallels to both embryonic development and wound repair in vertebrate organisms.

A.2 Why computer models? Advantages and limitations
Every day, experimental research in the life sciences generates vast amounts of data. New tools and technologies continue to increase the available information on the physical and molecular underpinnings of complex biological systems. Better-resolved measurements, both in space and time, provide a more detailed view of cellular function. In parallel, our ability to integrate the detailed mechanisms that govern molecular, cellular, tissue, organ, and animal dynamics becomes progressively more limited, and it is challenging to formulate coherent and comprehensive hypotheses about how living systems work (Mazzocchi, 2008). It is also
increasingly difficult to design and conduct experiments that test hypotheses bridging molecular dynamics, cellular behaviours, and the generation of tissue architecture. For these reasons, the last 15-20 years have witnessed an explosion in the application of mathematical models to study complex biological systems. In combination with advances in computer science and engineering, mathematical models enable testing a wide range of hypotheses in silico by conducting many variants of complex experiments that would be extremely costly, time-consuming, and technically-challenging in vivo.

Epithelial tissues are excellent examples of complexity spanning the molecular, cellular, and tissue scales. Epithelia serve a barrier function by forming molecularly polarized and mechanically integrated sheets that can serve adsorptive and secretory functions. Tissue form and function are intimately linked, and thus, epithelia can be shaped as sheets, tubes, or branching trees. The acquisition of complex architectures occurs while preserving tissue integrity through coordinated cell movements and rearrangements. Coordinated cell movements are mediated by the remodelling of cytoskeletal and adhesive structures to generate mechanical forces that facilitate cohesive migration (Wood et al., 2002; Gaggioli et al., 2007; Hidalgo-Carcedo et al., 2011; Abreu-Blanco et al., 2012; Hunter et al., 2015; Matsubayashi et al., 2015), and mechanical forces feed back on the organization of molecular networks inside the cell (Effler et al., 2006; Sawada et al., 2006; del Rio et al., 2009; Fernandez-Gonzalez et al., 2009; Yonemura et al., 2010; Pines et al., 2012; Weber et al., 2012). The strong interdependence between mechanical and molecular signals makes it difficult to interpret the relative contribution of different cues to the shaping of epithelial tissues.

Mathematical models can disentangle the complex relationship between molecular networks and physical signals in epithelial morphogenesis. This can be achieved, for instance, by exclusively including molecular or mechanical signals in a model and measuring the resultant phenotypes. Similarly, it is possible to distinguish between the specific roles of different molecular components (e.g. cytoskeletal vs. adhesive). There are other advantages to the use of mathematical models (Brodland, 2015). Models are tremendously useful for their predictive power and the flexibility that they allow when conducting in silico
experiments. When a biological system is represented by a set of equations, the system can be investigated even when some of its parameters are unknown. System parameters can be added, removed, or modified to make predictions about their roles. Therefore, models are excellent tools to generate hypotheses and to prioritize *in vivo* experiments and reduce associated costs. Models can also be used to quantitatively analyze data by determining the parameter values that provide the best fit for experimental results. Mechanical models, for instance, have been used to infer the viscosity, elasticity, and stiffness of epithelia from different experimental manipulations, such as laser ablation (Hutson et al., 2003; Fernandez-Gonzalez et al., 2009), atomic force microscopy (Lau et al., 2015), or optical tweezers (Bambarekar et al., 2015). The main limitation of mathematical models is that, unlike wet lab experiments, models cannot prove or disprove hypotheses. Models show conditions that are consistent with a particular hypothesis, but need to be experimentally validated. Therefore, models are excellent tools for discovery and data interpretation, but their utility is limited for hypothesis testing.

### A.3 Vertex models: a primer

Several modelling frameworks have been applied to the study of epithelial tissues (for a review, see (Fletcher et al., 2013)). In some approaches, including finite elements (Clausi, 2001) or the cellular Potts model (Graner and Glazier, 1992), cells evolve on a grid, where they can only take on a discrete number of positions. While “grid methods” can recapitulate tissue-wide movements, they often fail at capturing the complex cell morphologies and changes thereof sustained by epithelial cells. In contrast, vertex models allow movements that are not restricted to pre-specified lattice positions, and they can be extended to faithfully capture the curvature and dynamics displayed by epithelial cells *in vivo* (Brodland et al., 2014).

Vertex models have been extensively used to investigate epithelial morphogenesis. In a vertex model, each cell is represented by a polygon (Nagai and Honda, 2001). Cells are combinations of edges (the sides of the polygons) and nodes (points where three or more polygons meet) (Figure A1A-C). Most vertex models only include the apical surface of the
Figure A1: Representing cells as nodes and edges.

(A) An epithelial monolayer in the Drosophila embryo. Cells express E-cadherin:GFP. Scale bar, 10 µm. Anterior left, dorsal up. (B) Polygons (green) can be used to represent the cells in (A). (C) Zoom into the white box in (B). Vertices where three or more polygons meet constitute a node (cyan). Interfaces between two cells are represented by edges (yellow).
cell, or a cross-section along the apical-basal axis (Sherrard et al., 2010), effectively treating cells as two-dimensional entities, and thus reducing the data necessary to set up the model and the associated computational complexity. However, new microscopy techniques enable detailed visualization of living cells in three dimensions, and computational power is increasingly affordable. Thus, vertex models have recently been used to investigate morphogenesis in three dimensions, for instance, in the transition from a two-dimensional epithelial primordium to a three-dimensional tube during eggshell appendage formation in *Drosophila* (Osterfield et al., 2013; Du et al., 2014).

In a vertex model, a set of rules governs the movement of each vertex. Rules are expressed as differential equations that track changes in vertex position (see Equation A.1 below). Cells are assumed to be in a medium in which viscosity, $\mu$, dominates over the inertial forces that tend to maintain a body in motion. The ratio of inertial to viscous forces is known as Reynolds number (Purcell, 1977). At a low Reynolds number, and assuming a low speed of motion that does not cause turbulence and increased resistance to movement, the forces acting on vertex $i$, $F_i$, are opposed by the drag force, proportional to the velocity of the vertex. At equilibrium, the sum of forces acting on a given vertex equals the drag force:

$$F_i = \mu \frac{dr_i}{dt}.$$  \hspace{1cm} (A.1)

where $\mathbf{r}_i$ represents the position of vertex $i$. Equation A.1 is a motion equation. Vertex models include as many motion equations as there are vertices in the system. By specifying $F_i$ as a sum of different factors (*e.g.* actomyosin contractility, cell-cell adhesion, or pressure), it is possible to investigate the contribution of different physical forces to the system (Box A1). Conversely, based on changes in vertex position measured from live imaging experiments, it is possible to infer the forces that act on the vertices (Brodland et al., 2010; Chiou et al., 2012; Brodland et al., 2014).

Vertex models can also integrate biochemical information. For example, if one includes actomyosin contractility as a force that governs the movement of vertices, the value assigned to contractile force ($\Gamma_\alpha$, Box A1) could be represented by the concentration of myosin motors.
Box A1: Defining motion equations.

In vertex models, motion is often defined in terms of an energy function, $U$, that represents the energy stored in the cells (Fletcher et al., 2014). The force acting on a vertex is related to spatial differences in energy around the vertex, which can be mathematically represented by the negative derivative of the energy function with respect to the position of the vertex (Farhadifar et al., 2007). The use of energy-based motion equations facilitates the investigation of energy minimization problems in which the system relaxes towards equilibrium (e.g. after a cell divides or dies, or if a junction is severed). In this case, optimization algorithms can be used to find the lowest energy cell configuration to evolve into based on the value of $U$. Energy-based motion equations can include the tendency of cells to maintain a constant volume (or area); the presence of a contractile cortical cytoskeleton that minimizes the perimeter of the cell; and the cell-cell adhesion system that promotes an increase in contact length between two cells. Thus, a typical energy function (Farhadifar et al., 2007; Fletcher et al., 2014) is:

$$
U = \sum_{\text{for every cell } \alpha} \left( K_{\alpha} (A_{\alpha} - A_{\alpha_0})^2 + \Gamma_{\alpha} P_{\alpha}^2 \right) + \sum_{\text{for every junction } jk} A_{jk} l_{jk},
$$

where $K_{\alpha}$ is the elastic modulus of cell $\alpha$, $A_{\alpha}$ is the cell area, $A_{\alpha_0}$ is the preferred cell area (when the cell sustains no stress), and $\Gamma_{\alpha}$ is the cortical contractility, representing the force exerted by myosin motors on actin filaments in the cytoskeletal belt around the cell perimeter, $P_{\alpha}$; $A_{jk}$ is the junctional tension at an edge connecting vertices $j$ and $k$, and $l_{jk}$ is the length of that edge. The first term of the energy equation is a sum over all the cells in the system. The first factor in the sum represents the incompressibility of the cell: changing the cell area with respect to the preferred or rest cell area will result in an increase in cell energy, therefore causing the system to deviate from an equilibrium configuration in which $U$ is minimized. The second factor represents contractile forces generated by the actomyosin cortex around the cell perimeter, which acts to minimize the cell surface. Increasing contractility or surface area results in greater energy. The second term of Equation A.2 is a sum of line tensions over all the cell-cell contacts. The line tension represents the tendency of a cell-cell contact to decrease its length. When line tension is positive, cytoskeletal contractility dominates over cell adhesion and contact length will decrease. Conversely, when line tension is negative, cell adhesion dominates and contacts become longer.
at the cell cortex multiplied by the force generated per motor (Wang et al., 2012; Lan et al., 2015). Similarly, if adhesion plays an important role in the system, one could define the line tension at cell-cell contacts ($A_{jk}$, Box A1) as the difference between actomyosin-contractility at that contact (calculated as above) and adhesive forces, estimated by the concentration of E-cadherin, a core cell-cell adhesion protein in epithelia, multiplied by the adhesive force of cadherin-cadherin interactions (Gemp et al., 2011). When biochemical information is included, the evolution of the different molecular species should be monitored over time with the help of differential equations.

Differential equations are used to model protein dynamics and protein-protein interactions (Lauffenburger and Linderman, 1995; Mogilner et al., 2006). In the case of “well-stirred” systems, in which molecules are uniformly distributed, ordinary differential equations are sufficient to track changes in the concentration of molecules, as all changes happen in time, and not in space (Box A2 and Figure A2). But, physical parameters can affect molecular interactions in the cell. Of particular importance is diffusion, or the random thermal motion of particles along concentration gradients. Diffusion is the dominant physical transport process by which molecules approach one another in binding reactions. If reactants are uniformly distributed throughout the “reactive” volume, binding is not diffusion-limited. In contrast, in systems in which molecules are produced or degraded at specific locations, diffusion can play a critical role, and partial differential equations are necessary to model changes in molecular concentration, both in time and space (Box A3).

### A.4 Modelling complex morphogenetic processes: dorsal closure

The development of realistic vertex models requires considerable data. Furthermore, experimental tests must ensue to validate the results of the model. For these reasons, many studies that apply vertex models to investigate epithelial morphogenesis focus on *Drosophila*, as the fruit fly allows live imaging of developmental processes, as well as genetic, pharmacological and biophysical manipulations. Vertex models have recently been applied to the analysis of oogenesis (Koride et al., 2014); eggshell formation (Osterfield et al., 2013; Osterfield et al., 2015); anterior-posterior axis elongation (Rauzi et al., 2010;
Box A2: Modelling “well-stirred” systems: ordinary differential equations.

The use of ordinary differential equations to model biochemical reactions is based on the application of the law of mass action [95]. The law of mass action indicates that the rate of a chemical reaction is proportional to the product of the concentrations of the reactants. In other words, the probability of a biochemical reaction is proportional to the probability of collision between the reactants, which itself is a function of the reactants’ concentrations. As an example, consider a simplified version of the assembly of myosin into minifilaments (Figure A2). We will assume that myosin molecules can be present in a cytoplasmic pool (at a concentration $m_c$), or as part of minifilaments on the cortex (at a concentration $m_f$); that cytoplasmic molecules are activated by phosphorylation by the kinase Rho-kinase (Rok) to form minifilaments; and that minifilament disassembly is a first-order decay process dependent exclusively on $m_f$. We can monitor the evolution of $m_f$ using this equation:

$$\frac{dm_f(t)}{dt} = k_a R(t) m_c(t) - k_d m_f(t), \quad (A.3)$$

where $R$ represents the concentration of Rok. The left-hand side of Equation A.3 is the derivative, or change over time, of the concentration of myosin in minifilaments. The right-hand side of the equation consists of two terms. The first term represents the rate of myosin assembly into minifilaments, which is proportional to the concentrations of cytoplasmic myosin and Rok, modulated by a proportionality factor, $k_{as}$ or assembly rate constant. The second term represents the rate of myosin dissociation from minifilaments, proportional to the concentration of cortical myosin, and with disassembly rate constant $k_d$. Similar equations can be written for the evolution of $m_c$ and $R$. The solution of the system of three equations, generated using software tools, can be evaluated at time $t$ to obtain the concentration of the three molecular species at that specific time. Note that, although in Equation A.3 the values of $k_a$ and $k_d$ are not time-dependent, they could be, thus modelling temporal transitions in the rate of myosin minifilament assembly or disassembly. Importantly, more complex dynamics can be applied to investigate protein-protein interactions; for instance, Michaelis-Menten kinetics can be used to model enzymatic reactions [9696].
Figure A2: A simplified model of myosin minifilament assembly and disassembly.

Left, group of four cells. Right, magnified view of the black box. Cytoplasmic unassembled myosin motors (pink, $m_c$), cortical assembled myosin minifilaments (green, $m_f$), Rok (blue), and cell membrane (yellow) are shown. Note that Rok can be found associated with the cortex, but in this simplified model we assume that it is exclusively cytoplasmic. The assembly of myosin filaments occurs at rate constant $k_a$; myosin minifilament disassembly into subunit monomers occurs at rate constant $k_d$. 
Box A3: Modelling with diffusion: partial differential equations.

Diffusion affects chemical reactions by altering the concentration of the participating molecules. Molecular flux, defined as the movement of molecules caused by diffusion, occurs from high to low concentrations, with a magnitude that is proportional to the concentration gradient or spatial derivative of the concentration profile. Mathematically, this is represented by Fick’s 1st law:

$$\phi(x, t) = -D \frac{\partial c(x,t)}{\partial x} = -D \nabla c,$$

where $\phi(x, t)$, the diffusive flux, is a vector representing the net number of molecules that cross a unit area per unit time at location $x$; $c$ is the concentration of the molecule that diffuses, and $D$ is a proportionality constant known as the diffusion coefficient. $\nabla$ indicates the spatial derivative along all spatial dimensions, and thus, in three-dimensional space,

$$\nabla = \frac{\partial}{\partial x} \mathbf{i} + \frac{\partial}{\partial y} \mathbf{j} + \frac{\partial}{\partial z} \mathbf{k},$$

with $\mathbf{i}$, $\mathbf{j}$, and $\mathbf{k}$ unit vectors along the $x$, $y$, and $z$ dimensions, respectively. $\nabla c$ is referred to as the gradient of $c$, and it represents a vector pointing in the direction of maximum change of $c$. Fick’s 1st law indicates that molecules will move at a speed determined by the diffusion coefficient $D$, in the direction in which $c$ decreases (thus the negative sign) most rapidly.

The continuity equation relates changes in flux across space (i.e. changes in the number of molecules that diffuse in and out of a volume) to changes in concentration over time:

$$\frac{\partial \phi(x,t)}{\partial x} = - \frac{\partial c(x,t)}{\partial t} \text{ or } \nabla \phi = - \frac{\partial c}{\partial t},$$

Intuitively, if the incoming flux is greater than the outgoing flux (i.e. the flux decreases in space), the concentration will increase, and vice versa. Substituting Fick’s 1st law into the continuity equation, one can derive the diffusion equation, also known as Fick’s 2nd law:

$$\frac{\partial c(x,t)}{\partial t} = D \frac{\partial^2 c(x,t)}{\partial x^2} \text{ or } \frac{\partial c}{\partial t} = D \nabla^2 c,$$

where $\nabla^2$ is the Laplacian operator or second spatial derivative. The diffusion equation can track changes in $c$ over time for molecules that are not uniformly distributed in space. But molecular flux is also affected by chemical reactions. Therefore, when modelling changes in molecule concentration in the presence of diffusion, it is important to also include reaction terms. As an example, one can extend the scenario described in Box 2, such that myosin is produced in the proximity of the cell nucleus and diffuses towards cell-cell contacts. In that case, the equation that governs the change in cytoplasmic myosin, $mc$, is given by:

$$\frac{\partial mc(x,t)}{\partial t} = D \frac{\partial^2 mc(x,t)}{\partial x^2} + k_d m_t(x,t) - k_R R(x,t) mc(x,t),$$

which includes both diffusion and reaction terms. Notice that, with this equation, we monitor the concentration of cytoplasmic myosin both in time ($t$) and space ($x$). The first reaction
term represents the disassembly of myosin from minifilaments, which produces cytoplasmic myosin; and the second term represents the interaction between cytoplasmic myosin and Rok to add myosin to minifilaments.
Figure A3: Dorsal closure in *Drosophila* embryos.

(A) Cartoons showing the sealing of an opening on the dorsal surface of the *Drosophila* embryo epidermis. An actomyosin purse string (magenta), and constricting and apoptotic amnioserosa cells (light grey) contribute to this process. (B-B’) *Drosophila* embryo in the initial stages of dorsal closure. E-cadherin, a cell-cell adhesion marker, is shown in green; phalloidin, a marker for filamentous actin, is magenta. Scale bar, 50 µm. Anterior left. (B’) Magnified view of the white box in (B). Arrowheads indicate actin protrusions extending from the leading edge of the epidermis out of the purse string. Scale bar, 5 µm.
Collinet et al., 2015; Lan et al., 2015) and mesoderm invagination (Conte et al., 2012; Hocevar Brezavscek et al., 2012; Polyakov et al., 2014; Rauzi et al., 2015) in the embryo; cell division and growth (Farhadifar et al., 2007; Gibson et al., 2011; Smith et al., 2012), cell packing (Farhadifar et al., 2007; Aigouy et al., 2010; Staple et al., 2010) and boundary formation (Landsberg et al., 2009; Aliee et al., 2012; Umetsu et al., 2014a) in wing imaginal discs (sacs of epithelial cells at larval and pupal stages that will form the adult wings); or cell sorting in the retina (Hilgenfeldt et al., 2008). Here, we discuss the application of vertex models to dorsal closure, a morphogenetic process in the Drosophila embryo that involves complex molecular dynamics, multiple cell types and behaviours, and dramatic changes in tissue architecture, while displaying many analogies with other developmental and repair processes.

During dorsal closure, a discontinuity on the dorsal epidermis is sealed through the lateral migration, spreading and fusion of two cell sheets (Figure A3A) (Campos-Ortega and Hartenstein, 1985). Similar processes drive ventral enclosure in C. elegans (Williams-Masson et al., 1998), zebrafish epiboly (Behrndt et al., 2012), eyelid closure in mammals (Heller et al., 2014), or wound healing in embryos (Martin and Lewis, 1992; Brock et al., 1996; Wood et al., 2002) and in epithelial monolayers in vitro (Bement et al., 1993; Tamada et al., 2007). The epidermal discontinuity is bridged by the amnioserosa, an epithelial monolayer. During dorsal closure, the two lateral epithelial sheets that form the epidermis move over the amnioserosa and meet at the dorsal midline of the embryo. Several cellular behaviours contribute to dorsal closure: the leading edges of the advancing epidermal sheets become enriched with actomyosin, forming a contractile supracellular cable that minimizes the perimeter of the epidermal discontinuity (Young et al., 1993; Edwards et al., 1997; Kiehart et al., 2000); cellular protrusions from opposite sides of the epidermis interdigitate and zip the dorsal opening at its corners (Jacinto et al., 2000; Hutson et al., 2003); amnioserosa cells constrict their apical cell surfaces in a pulsatile manner, thus pulling centripetally on the epidermal sheets (Solon et al., 2009; Blanchard et al., 2010; David et al., 2010); and apoptosis-induced delamination of a subset of amnioserosa cells also facilitates the overall reduction in area of the dorsal opening (Toyama et al., 2008). In spite of the
detailed characterization of the cellular behaviours involved in dorsal closure, it is still unclear how they are coordinated.

Computer modelling can help understand the synergies between different cellular behaviours during epithelial morphogenesis. A number of experimental approaches, including genetic manipulations (Jacinto et al., 2002), as well as quantitative image analysis (Blanchard et al., 2010) and laser ablation (Kiehart et al., 2000; Hutson et al., 2003), have been established to investigate the relative contribution of different cell behaviours to dorsal closure. Computer models provide an opportunity to integrate experimental findings by isolating individual cell behaviours and putting them back together in different combinations, at multiple spatial and temporal scales. Thus, computational models facilitate the generation of hypotheses about the interplay between different cellular behaviours that can then be experimentally tested.

During the early stages of dorsal closure, amnioserosa cells undergo apical area oscillations (Solon et al., 2009). Pulsed contractions have been observed in other tissues and animals (Martin et al., 2009; Roh-Johnson et al., 2012; Lau et al., 2015). Whether oscillatory behaviours are cell-autonomous or not is a matter of controversy that has been explored using vertex models. During dorsal closure, amnioserosa cells pulse predominantly in anti-phase with their neighbours (Solon et al., 2009). Amnioserosa oscillations are driven by the cyclical assembly, contraction, and disassembly of medial-apical actomyosin networks (Blanchard et al., 2010; David et al., 2010). Computer modelling of dorsal closure suggested that the pulsatile contractility of amnioserosa cells was not cell-autonomous (Solon et al., 2009), and had to be linked to pulsing in adjacent cells to reproduce the anti-phase cell area oscillations observed in vivo. These results were consistent with data showing that wounding amnioserosa cells inhibits the oscillatory behaviours of the wounded cells, and also those of their immediate neighbours (Solon et al., 2009; Saravanan et al., 2013). Pulses of contraction in the amnioserosa were modelled as a sigmoid curve (Hill function) with respect to cell deformation (Solon et al., 2009). Thus, in the model, amnioserosa cells experienced a contractile force when their apical surface area increased beyond a critical value. This approach led to pulsed contractions of amnioserosa cells predominantly in anti-phase between neighbours, suggesting that pulsing in the amnioserosa is caused by a deformation-
mediated mechanism, a prediction that has yet to be tested. A limitation of this model, according to Jayasinghe et al. (Jayasinghe et al., 2013), is that the critical deformation that induced cell contraction was too large to be realistic, as it required an elastic strain of approximately 3. A critical strain of 3 implies that, to induce a contractile response, the increase in length of cell-cell contacts should be 300% of the rest length of the contacts, and there are no materials that are linearly elastic to that strain (Jayasinghe et al., 2013). Spherical lipid bilayers, for instance, can only be deformed by 2-4% before they rupture (Zhelev and Needham, 1993). Other studies using vertex models have proposed that small deformations can drive tissue oscillations (Wang et al., 2012; Jayasinghe et al., 2013) if cell pulsing is at least in part cell-autonomous (Jayasinghe et al., 2013). Supporting this idea, when an amnioserosa cell is isolated from its neighbours by severing all contacts by laser ablation, the apical area of the cell decreases rapidly if the cell was in a contraction cycle before ablation (Jayasinghe et al., 2013). But cells that were expanding immediately before isolation maintain their apical surface for a few seconds before the onset of apical constriction, suggesting that the oscillatory behaviours of amnioserosa cells are at least in part linked to the active assembly and disassembly of contractile networks within the cell, and not solely to the behaviour of neighbouring cells. In the follicle cells of the Drosophila egg chamber, where the basal surface undergoes actomyosin-driven, pulsed oscillations, inhibition or constitutive activation of actomyosin contractility in a mosaic pattern did not disrupt the oscillations of neighbouring, wild-type cells, indicating that oscillations are cell-autonomous (He et al., 2010). In this context, cell-extracellular matrix adhesion was critical for pulsed contractions (He et al., 2010; Koride et al., 2014). Further experiments will be necessary to determine whether pulsatile behaviours in the amnioserosa are cell-autonomous or not, or if they are driven by a combination of cell-autonomous and non-autonomous forces (Sokolow et al., 2012). During Drosophila mesoderm invagination (Martin et al., 2009; Martin et al., 2010) and axis elongation (Rauzi et al., 2008; Fernandez-Gonzalez and Zallen, 2011; Sawyer et al., 2011), pulsed contraction and expansion of the apical surface is cell-autonomous, and adhesion to neighbouring cells makes contractile pulses anisotropic. Reducing cell-cell and cell-extracellular matrix adhesion in the amnioserosa, or using optogenetic approaches to locally disrupt actomyosin contractility without causing a wound
will increase our understanding of the autonomy of pulsatile behaviours during dorsal closure.

Pulsatile behaviours drive apical constriction of amnioserosa cells and promote dorsal closure. Amnioserosa oscillations switch from a non-productive mode, in which neither the cells nor the tissue display an overall reduction of their surface area, to a mode in which the expansion of amnioserosa cells is to a monotonically decreasing maximum area, and both the cells and the tissue contract (Solon et al., 2009; Blanchard et al., 2010). Amnioserosa oscillations dampen over time, as the cells constrict apically. The reduction in the amplitude of cell pulsing occurs from the periphery of the amnioserosa towards the centre of the tissue. The mechanism that ratchets the pulsed contractions of amnioserosa cells remains unknown. Vertex modelling suggested that contraction of the actomyosin cable at the leading edge of the advancing epidermis generates compressive forces that restrict the ability of amnioserosa cells to relax after a cycle of contraction (Solon et al., 2009). Consistent with this, embryos in which the actomyosin cable does not form, display sustained oscillatory behaviours in the amnioserosa with no sign of dampening. However, a vertex model in which the actomyosin cable was the only ratchet, implemented by reducing the rest length of the cable over time, failed to suppress pulsatile behaviours and induce tissue contraction beyond the most peripheral layer of amnioserosa cells (Wang et al., 2012). The model included radial elastic spokes connecting cell vertices to the cell centre in amnioserosa cells to represent medial-apical actomyosin networks. Resistance to compression by the radial spokes, and therefore by medial-apical actomyosin networks, could explain why the actomyosin cable is not sufficient to promote amnioserosa cell contraction in the model (Wang et al., 2012), although laser ablation data suggest that the main role of media-apical actomyosin networks is to promote contraction rather than resisting it (Ma et al., 2009; Jayasinghe et al., 2013). An internal ratchet, representing the junctional actomyosin cortex of each amnioserosa cell (Blanchard et al., 2010), was modelled by a progressive reduction in the rest length of each cell junction and radial spoke. Under those conditions, the internal ratchet alone was able to reproduce the attenuation of amnioserosa cell pulses, following a spatial pattern reminiscent to that observed in vivo, and not requiring the actomyosin cable (Wang et al., 2012). Thus, remodelling of the actomyosin cortex may provide the ratchet, and the actomyosin cable may
reinforce it (Martin et al., 2009; Wang et al., 2012). Independent disruptions of contractility in the cortex of amnioserosa cells or the cable at the leading edge of the epidermis will help to determine the relative contribution of different actomyosin networks to ratchet pulsed contractions.

Additional mechanisms can dampen the oscillations of amnioserosa cells. In vivo experiments showed that amnioserosa cell pulsation is controlled by the polarity proteins Par-3 and aPKC (David et al., 2010). aPKC promotes the disassembly of actomyosin networks in the medial-apical surface of amnioserosa cells by acting upstream of cytoskeletal regulators. aPKC also recruits Par-3 to the medial-apical surface. By binding Par-3, aPKC is temporarily sequestered from its cytoskeletal targets. After binding, aPKC phosphorylates Par-3. Par-3 is then displaced from the apical surface, and aPKC is released and can act again on cytoskeletal regulators, thus promoting network disassembly. Stabilization of Par-3-aPKC complexes on the medial-apical surface of amnioserosa cells by an unknown mechanism could suppress cell pulsing by sequestering aPKC and facilitating the assembly and continuous contraction of actomyosin networks to drive apical constriction (David et al., 2013). To test this hypothesis, a vertex model was used in which myosin dynamics were tracked using an ordinary differential equation (Wang et al., 2012; David et al., 2013) (Box 2). In this framework, the force sustained by the spokes connecting the cell cortex to the cell centre (the medial-apical actomyosin network) was dependent on the levels of myosin on the spoke. Myosin levels were given by a kinetic equation that included myosin production and assembly, and myosin disassembly (Wang et al., 2012). To simulate Par-3-aPKC complex stabilization on the medial-apical cell surface, the production of myosin was increased and the rate constant of myosin disassembly was decreased (David et al., 2013). Under these conditions, cell pulsation ceased, and amnioserosa cells partially constricted their apical surfaces. Together, these results suggest that biochemical control of myosin dynamics may regulate the transition between non-productive pulsed contractions and apical constriction. It would be interesting to investigate if biochemical regulation alone can induce apical constriction of amnioserosa cells when mechanical ratchets (internal or external) are not present. Furthermore, the mechanisms that would lead to the stabilization of Par-3-aPKC interactions on the medial-apical surface of amnioserosa cells are unknown. It has been
proposed that the reduction in surface area as cells constrict apically could cause an increase in Par-3 concentration that inhibits aPKC (David et al., 2013). Extending the model to track the dynamics of Par-3 and aPKC, and their interplay with myosin, may provide additional mechanistic information about how the transition from non-productive to productive myosin pulses is governed during dorsal closure.

Finally, what is the role of protrusive activity during dorsal closure? Cells at the leading edge of the advancing epidermis extend filopodia, or finger-like, actin-based protrusions, during dorsal closure (Figure 3B). Filopodia from opposite sides of the epidermis can interdigitate in a process referred to as zipping. Recent work suggests that zipping is the critical step to seal the discontinuity in the epidermis (Lu et al., 2015). Zipping is promoted by apical constriction and cell delamination in the amnioserosa, and by contraction of the actomyosin cable at the leading edge of the epidermis. These processes bring together the two epidermal sheets, thus facilitating the establishment of points of cell-cell adhesion across the amnioserosa. Vertex models that include the contribution of zipping to dorsal closure have not been developed, presumably because the dynamics of actin protrusions are complex (Jacinto et al., 2000; Millard and Martin, 2008), and building a model that can provide new mechanistic information may require too many unknown parameters. However, protrusive activity has been modelled during wound repair in epithelial monolayers in culture (Brugues et al., 2014). In this context, wound closure is driven by the collective migration of the cells adjacent to the wound into the wounded region (Bement et al., 1993). Cell movement is coordinated by an actomyosin purse string that forms at the leading edge of the cells adjacent to the wound. Cells adjacent to the wound also extend actin-based protrusions that facilitate cell crawling towards the interior of the wound by establishing contacts with the substrate (Fenteany et al., 2000). Using a vertex model in which cells extended protrusions at the wound edge with a probability twice greater than elsewhere in the epithelial sheet, it was shown that protrusion-based cell crawling and actomyosin cable contractility were both individually sufficient to drive wound repair. However, the model could only recapitulate the pattern of traction forces observed in cells when actin protrusions and the actin cable were combined, and the actin cable contracted non-uniformly at the wound edge. Notably, in embryonic wound repair, significant (albeit slower and/or incomplete) wound closure can
occur in mutants in which the actin cable does not form or in which cells cannot assemble actin-based protrusions (Wood et al., 2002; Abreu-Blanco et al., 2012). During dorsal closure, leading-edge epidermal cells expressing dominant-negative Cdc42 (Harden et al., 1999) or mutant for the JNK kinase (Glise et al., 1995) do not form protrusions, and the embryos display dorsal holes as a consequence of improper seam formation at the dorsal midline, incomplete closure and misalignment of corresponding stripes from opposite sides of the embryo (Jacinto et al., 2000). Whether actin-based protrusions serve adhesive, crawling or guidance roles (or all of the above) during dorsal closure remains to be investigated. Recent advances in the understanding of the actin regulators that control protrusive activity during dorsal closure (Homem and Peifer, 2009; Pickering et al., 2013; Nowotarski et al., 2014), as well as the development of image analysis tools for the automated tracking and quantification of protrusive structures from fluorescence microscopy images (Tsygankov et al., 2014; Barry et al., 2015) will provide sufficient information about actin protrusion dynamics to explore their relative contribution to epidermal sealing, both experimentally and using computer models.

A.5 Conclusion

Vertex models are useful to investigate both the mechanical and biochemical regulation of epithelial morphogenesis. The role of mechanical forces in embryonic development and tissue repair is increasingly recognized, and experimental methods to image, measure and manipulate the impact of cell mechanics on cell signalling in vivo continue to be developed. The amount of data quantifying the complex interplay between physical factors and cell communication and behaviour during epithelial morphogenesis is fast growing. The application of mathematical modelling approaches will be necessary to pre-screen interesting hypotheses in silico and facilitate the design of in vivo experiments. Computer models will therefore become the new lab aids for developmental, cellular, and molecular biologists; it is therefore critical for modern biologists to gain familiarity with the theoretical foundation behind these tools.
A.6 Acknowledgements

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Appendix B: Mechanical regulation of actin at resolving vertices

In preliminary experiments, I measured the dynamics of actin assembly at new interfaces (Figure B1A) in embryos expressing both GFP:MoesinABD, a filamentous actin reporter (Kiehart et al., 2000), and Gap43:mCherry, a plasma membrane marker (Martin et al., 2010) (Figure B1B). Similar to the elongation of new DV edges (Yu and Fernandez-Gonzalez, 2016; Chapter 2), the assembly of actin occurred in pulses, both at interfaces (Figure B1C, red solid line) and at the TCJs at the ends of the interface (Figure B1F, red lines). Actin pulses at interfaces occurred every 116.62±6.72 s, consistent with previously published cycles of edge elongation and of the apical area oscillation in the cells adjacent to the new interface (Fernandez-Gonzalez and Zallen, 2011; Sawyer et al., 2011; Yu and Fernandez-Gonzalez, 2016; Chapter 2). To examine whether the pulsatile elongation of new interfaces was associated with the pulsed assembly of actin, I compared the changes in length of the nascent DV edge to the changes in actin fluorescence at the junction (Figure B1C). Using cross correlation, a measurement of signal similarity, I found that the correlation between changes in edge length and actin fluorescence increased dramatically when I shifted the actin curves by 9±6 s forward in time (Figure B1D-E), indicating that pulses of new DV edge assembly were preceded by bursts of actin fluorescence. I took a similar approach to investigate the pulsed actin accumulation at TCJs flanking elongating interfaces, and found that actin bursts at TCJs also preceded pulses of interface elongation by 12±6 s (Figure B1F-H).
Figure B1: The oscillatory assembly of new interfaces is positively correlated with the pulsatile accumulation of actin.

(A) The anisotropic tension sustained along the anterior-posterior axis of the embryo polarizes the assembly of actin filaments at the nascent interface, and may also promote actin polymerization at its associated tricellular vertices. (A') If actin polymerization is regulated differently at these domains, then actin dynamics at the interface should be analyzed independently of actin activity at tricellular vertices. (B) Kymographs of nascent edge elongation during axis elongation in an embryo expressing gap43:mCh (magenta, top; greyscale, middle) and GPF:moesinABD (green, top; greyscale, bottom). Scale bar, 15 s. The interface is rotated by 90° with respect to (A). Anterior down, dorsal left. (C,F) Rates of change for edge length (blue) and actin fluorescence at interfaces (C; red solid line) or vertices (F; anterior vertex, red dashed line; posterior vertex, red dotted line) of the nascent interface shown in (B). Rate of change was calculated with respect to t + 60 s. (D,G) Changes in correlation between edge length and actin fluorescence at interfaces (D) or vertices (G) of the nascent interface shown in (B) when the edge length signal was shifted in time in 15-second increments. Arrowheads indicate the correlation minima (blue) or maxima (red) closest to 0 s shift. (E,H) Distribution of time shifts required to obtain the minimum (blue) and maximum (red) correlations in all 21 junctions shown in (E) or all 42 vertices shown in (H).
Figure B2: Mechanical tension promotes actin accumulation at resolving DV interfaces.

(A-B) Cells expressing GFP:Utrophin, a marker of filamentous actin, in a sham-irradiated embryo (A) or an embryo in which the cells anterior and posterior to the resolving vertex were UV-irradiated to induce their apical constriction (B). Asterisks show the irradiated cells. Anterior left, dorsal up. Scale bars, 5 µm. (C) Maximum fold-change in total actin fluorescence in controls (blue, n = 10 interfaces) and under increased tension along the AP axis (red, n = 14 interfaces). Maximum fold-change in fluorescence was calculated with respect to the time point when the nascent DV interface first exceeded 1 µm in length, considering the five minutes after that time point. Error bars, s.e.m.
Appendix C: Candidate screen for actin regulators

Table C1: Candidate screen for actin regulators involved in early *Drosophila* embryonic development.

Actin regulators ranked by relative expression levels at 2-4 hours of development according to the modENCODE project (Celniker et al., 2009), with their respective outcomes from germband extension assays conducted with the indicated Gal4 drivers. All assays were performed at 25°C. Bold indicates genes that yielded defects in germband extension. TRiP lines are accompanied by their respective 5-digit Bloomington stock numbers.

<table>
<thead>
<tr>
<th>Gene</th>
<th>mat67+mat15 ♀</th>
<th>mat67 ♀</th>
<th>mat67 ♂</th>
<th>mat15 ♀</th>
</tr>
</thead>
<tbody>
<tr>
<td>ssh</td>
<td>severe defect</td>
<td>defect</td>
<td>defect</td>
<td>no phenotype</td>
</tr>
<tr>
<td>chic</td>
<td>34523</td>
<td>severe defect</td>
<td>severe defect</td>
<td>severe defect</td>
</tr>
<tr>
<td>arpc4</td>
<td>41888</td>
<td>defect</td>
<td>defect</td>
<td>defect</td>
</tr>
<tr>
<td>rho1</td>
<td>27727</td>
<td>no phenotype</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rho1</td>
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<td>defect</td>
<td></td>
<td></td>
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<tr>
<td>capt</td>
<td>33010</td>
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<tr>
<td>ctp</td>
<td>44044</td>
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<tr>
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<td>31582</td>
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<td>39034</td>
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<tr>
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<td>severe defect</td>
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<td></td>
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<tr>
<td>form3</td>
<td>32398</td>
<td>no phenotype</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Legend:
- **severe defect**: embryos were too fragile to be handled without bursting
- **defect**: germband extension assays showed defective axis elongation
- **no phenotype**: no obvious difference compared to controls
- **under low-level magnification**
Figure C1: Diaphanous depletion results in defects in germband extension.

(A-G) Images depicting the progression of germband extension in control embryos (A) and in embryos in which one of the six Drosophila formins had been downregulated by RNAi: capu (B), DAAM (C), fhos (D), frl (E), form3 (F), and dia (G). White arrowheads delimit the germband. Yellow arrowheads indicate the dorsalmost point of the cephalic furrow. Anterior left, dorsal up. Scale bars, 100 µm. (H-M) The degree of extension of the germband along the dorsal surface of the embryo in controls (blue) and upon downregulation of each of the six Drosophila formins (red): capu (H), DAAM (I), fhos (J), frl (K), form3 (L), and dia (M). Extension was measured with respect to the dorsalmost point of the cephalic furrow. Error bars, s.e.m.
Figure C2: Slingshot, a Cofilin phosphatase, is necessary for early embryogenesis in Drosophila.

(A-B) Images depicting germband extension in control embryos (A) and in ssh RNAi embryos (B). White arrowheads delimit the germband. Yellow arrowheads indicate the dorsalmost point of the cephalic furrow. Scale bars, 100 µm. (C) Percent extension of the germband along the dorsal surface of the embryo in controls (blue) and ssh RNAi embryos (red). Extension was measured with respect to the dorsalmost point of the cephalic furrow. Error bars, s.e.m. (D-E) Germband cells expressing Spider:GFP (green) and Myosin:mCherry (magenta) in controls (D) and in ssh RNAi embryos (E). Arrowheads indicate discontinuities in the epithelium. Scale bars, 10 µm.