ELUCIDATING THE ROLE OF INTEGRIN-MEDIATED ADHESION DURING ANIMAL MORPHOGENESIS

by

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Abstract

During the morphogenesis of multicellular organisms, cells undergo rearrangements and morphological changes to generate three dimensional structures and thus give rise to tissues and organs. Robust morphogenesis requires connections between neighbouring cells and to the extracellular environment in order to generate dynamic large-scale tissue rearrangements. Cellular adhesion to the extracellular matrix (ECM) is primarily mediated by the integrin family of adhesion receptors. Integrins are transmembrane heterodimeric receptors that bind ECM ligands extracellularly, and on their intracellular sides bind a diverse group of proteins known as the integrin adhesion complex (IAC). Integrins are involved in many fundamental biological processes and they contribute to animal development through two distinct mechanisms; first, via dynamic short-term adhesions such as those driving cell migrations and cellular rearrangements, and second via long-term stable adhesions such as those involved in tissue maintenance. Thus the activity of integrins needs to be finely regulated during morphogenesis. In this thesis, I aim to investigate the regulation of integrins during *Drosophila* morphogenesis in the context of both stable long-term adhesions, and short-term dynamic adhesions. First, I investigate the role of talin, a large scaffolding protein which provides a direct link between integrins and the actin cytoskeleton, in the context of muscle development during fly embryogenesis. Importantly, talin also functions as an integrin regulator by regulating the affinity of integrin for its extracellular ligands, a process known as inside-out activation. I describe results suggesting that the talin head domain reinforces and stabilizes the integrin adhesion complex by promoting integrin clustering in a mechanism distinct from its role in supporting inside-out activation.
Secondly I investigate a process known as dorsal closure (DC), an integrin-dependent dynamic morphogenetic event during fly embryogenesis that is characterized by rapid tissue movements, and the generation of biophysical forces. I find that integrins play a key role in regulating cell behaviours critical for DC via two main mechanisms: through modulating the localization of cell-cell adhesion proteins, and by regulating the dynamics of force-generating actomyosin machinery. Overall, my work highlights the diverse functions of integrins during development, and necessity for fine-tuning of their regulation during morphogenesis.
Preface

Chapter Two: A version of this work has been published as:


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For this publication Guy Tanentzapf and Stephanie Ellis conceived the experiments and designed the project. For the data presented in this thesis under the guidance of Stephanie Ellis I did all of the FRAP and talin/integrin recruitment experiments (Figure 2.1/2.2/2.3). I also contributed to the genetics for the wing clustering assays shown in Figure 2.3, and performed these experiments for Fig 2.5, including the quantifications. For the analysis of phenotypes (Figure 2.1/2.2) I contributed partially to these experiments, while being taught by Stephanie how to do them. I later independently measured the delay for rhea^{17} (Figure 2.2). I also performed all of the experiments measuring the recruitment of IAC components (Fig 2.4). Contributions of different authors are highlighted with their initials in bold (eg. Stephanie Ellis = SJE) The protein modeling was done by our collaborator, Ben Goult (Figure 2.5). I also contributed to the writing of the manuscript, a version of which appears here.

Chapter Three:

For this work I conceived of and executed the experiments with the assistance of Guy Tanentzapf. I was assisted in fly genetics, imaging and sample preparation by Kaitlyn Cramb and Tina Arndt. Katie Goodwin designed many of the analyses used, and ran some of the computations. For the K17E work, the majority of it was performed with Darius Camp who contributed significantly to fly genetics among other tasks.
Table of Contents

Abstract ................................................................................................................................. ii
Preface ................................................................................................................................. iv
Table of Contents ................................................................................................................. v
List of Figures ....................................................................................................................... viii
List of Fly Gene Names and Vertebrate Homologues ....................................................... xi
Acknowledgements ........................................................................................................... xii

CHAPTER ONE: Introduction ................................................................................................. 1
  1.1 Morphogenesis ............................................................................................................. 1
  1.2 Dorsal Closure (DC) ..................................................................................................... 3
    1.2.1 Overview of DC ...................................................................................................... 3
    1.2.2 Forces driving DC .................................................................................................. 4
    1.2.3 Signalling events contributing to DC .................................................................... 6
  1.3 Actomyosin Contractility in Morphogenesis ............................................................... 6
    1.3.1 Myosin .................................................................................................................. 7
    1.3.2 Actin ..................................................................................................................... 8
    1.3.3 Actomyosin dynamics ........................................................................................... 9
  1.4 Adhesion in Morphogenesis ......................................................................................... 9
    1.4.1 Cadherins ............................................................................................................. 10
  1.5 Cell Behaviours Driving Morphogenesis ..................................................................... 11
    1.5.1 Cell divisions ....................................................................................................... 11
    1.5.2 Cell intercalation .................................................................................................. 12
    1.5.3 Programmed cell death ....................................................................................... 13
    1.5.4 Contractile actomyosin rings ............................................................................... 14
    1.5.5 Apical constrictions ............................................................................................. 15
  1.6 Integrin Mediated Adhesion ......................................................................................... 16
    1.6.1 Integrin activation ................................................................................................ 17
    1.6.2 Integrin clustering ............................................................................................... 18
    1.6.3 Integrin structure ................................................................................................. 19
  1.7 Talin ............................................................................................................................. 20
    1.7.1 Talin structure ..................................................................................................... 20
  1.8 The Integrin Adhesion Complex during Development ............................................... 22
  1.9 Aims and Scope of This Thesis .................................................................................... 24
  1.10: Chapter 1 Figures .................................................................................................... 25

CHAPTER TWO: The Talin Head Domain Reinforces Integrin-Mediated Adhesion by Promoting Adhesion Complex Stability and Clustering ................................................. 29
  2.1 Chapter Two Introduction .......................................................................................... 29
  2.2 Chapter Two Results ................................................................................................ 33
    2.2.1 Integrin activation is not essential for fly embryogenesis .................................. 33
    2.2.2 A putative Rap-1 binding site in the talin head is dispensable for embryogenesis ......................................................................................................................... 36
2.2.3 The talin head is required for integrin-dependent morphogenesis and muscle attachment ................................................................. 36
2.2.4 rhea\textsuperscript{17} encodes a missense mutation in the talin head, G340E, required for talin function ........................................................................................................ 37
2.2.5 The G340E mutation abrogates integrin activation ....................................................................................................................... 39
2.2.6 The G340E mutation affects integrin clustering .......................................................................................................................... 39
2.2.7 rhea\textsuperscript{17} mutant embryos display defects in adhesion complex reinforcement ........ 41
2.2.8 The G340E mutation disrupts the interface between subdomains F2 and F3 in the talin head ........................................................................................................ 42

2.3 Chapter Two Discussion ........................................................................ 44
2.4 Chapter Two Materials and Methods ..................................................... 49
2.4.1 Molecular biology ............................................................................ 49
2.4.2 Fly stocks and genetics .................................................................... 49
2.4.3 Confocal immunofluorescence imaging and image analysis ............ 51
2.4.5 FRAP ............................................................................................... 53
2.5 Chapter 2 Figures: ................................................................................ 54

CHAPTER THREE: Investigating the Role of Integrin-Mediated Adhesion in Apical Cell Oscillations During DC ................................................................. 62

3.1 Chapter Three Introduction .................................................................. 62
3.2 Chapter Three Results ......................................................................... 66
3.2.1 Loss of basally localized integrins in the amnioserosa affects the amplitude of apical cell oscillations ................................................................. 66
3.2.2 Myosin speed is reduced in integrin mutants .................................... 67
3.2.3 Myosin polarization is unaffected by integrin loss ............................. 68
3.2.4 The subcellular localization of cadherin-based junctions is disrupted in integrin mutants ................................................................. 69
3.2.5 The kinetics of cadherin turnover are altered in integrin mutants ........ 70
3.2.6 Structure function mutations in integrin reveal a phenotypic variety of DC defects .................................................................................. 71
3.2.7 IntegrinYFP*N840A does not affect apical cell oscillations and is not required for cell movement during DC ........................................................................ 73

3.3 Chapter Three Discussion ...................................................................... 74

3.4 Chapter Three Materials and Methods .................................................. 77
3.4.1 Molecular biology ............................................................................ 77
3.4.2 Fly stocks and genetics .................................................................... 77
3.4.3 Confocal immunofluorescence imaging ........................................... 78
3.4.4 FRAP ............................................................................................... 80
3.4.5 Image analysis .................................................................................. 80

3.5 Chapter 3 Figures: ................................................................................ 82

CHAPTER Four: Discussion ......................................................................... 90
4.1 New Perspectives on Integrin Activation in Flies .................................. 90
4.2 A Role for Rap1 Direct Binding to the Talin Head .................................. 92
4.3 Integrins and Cadherins ....................................................................... 93
4.4 Overall Conclusions ............................................................................ 97
List of Figures

Chapter One

Figure 1.1 Three modules contributing to morphogenesis ........................................... 25
Figure 1.2 Dorsal Closure ................................................................................................. 26
Figure 1.3 Cell behaviours driving morphogenesis ......................................................... 27
Figure 1.4 The domain structure of the integrin heterodimer ......................................... 28

Chapter Two

Figure. 2.1 Integrin-binding to the talin head, but not integrin activation, is essential for talin-mediated integrin function in Drosophila development .............................. 54
Figure. 2.2 rhea\textsuperscript{17} encodes a hypomorphic talin protein which disrupts talin head function. ................................................................................................................. 56
Figure. 2.3 rhea\textsuperscript{17} disrupts integrin clustering. ........................................................ 58
Figure. 2.4. rhea\textsuperscript{17} disrupts adhesion complex reinforcement and adhesion consolidation. ................................................................................................................. 59
Figure. 2.5. G340 maintains an intermolecular interaction between F2 and F3 that couples their activity. ................................................................................................................. 61

Chapter Three

Figure 3.1 Loss of basal integrin results in decreased oscillation amplitude of apically constricting amnioserosa cells ................................................................. 82
Figure 3.2 Decreased oscillation amplitudes correlated with decreased myosin speeds. ................................................................................................................................. 83
Figure 3.3 The subcellular localization and kinetics of E-cadherin based junctions is disrupted in integrin mutants. ................................................................. 85
Figure 3.4 Structure–Function mutations in integrin reveal a phenotypic variety of DC defects .................................................................................................................. 87
Figure 3.5 IntegrinYFP*N840A does not affect apical cell oscillations and is not required for cell movement during DC ................................................................. 89
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABS</td>
<td>Actin binding sites</td>
</tr>
<tr>
<td>AJ</td>
<td>Adenosine-triphosphate</td>
</tr>
<tr>
<td>AS</td>
<td>Amnioserosa</td>
</tr>
<tr>
<td>ATP</td>
<td>Adherens junctions</td>
</tr>
<tr>
<td>CadGFP</td>
<td>E-Cadherin GFP</td>
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<tr>
<td>Crb</td>
<td>Crumbs</td>
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<tr>
<td>DC</td>
<td>Dorsal Closure</td>
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<tr>
<td>DPP</td>
<td>Decapentalplegic</td>
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<tr>
<td>E-Cadherin</td>
<td>Epithelial Cadherin</td>
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<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>ELC</td>
<td>Myosin essential light chains</td>
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<td>FA</td>
<td>Focal Adhesion</td>
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<td>FAK</td>
<td>Focal Adhesion Kinase</td>
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<td>FERM</td>
<td>Band 4.1/Ezrin/Radixin/Moesin</td>
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<td>FRAP</td>
<td>Fluorescence Recovery After Photobleaching</td>
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<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<td>IAC</td>
<td>Integrin Adhesion Complex</td>
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<tr>
<td>IBS</td>
<td>Integrin Binding Site</td>
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<tr>
<td>JNK</td>
<td>Jun-N-terminal kinase</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
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<tr>
<td>MTJ</td>
<td>Myotendious Junctions</td>
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<td>Non-muscle myosin II</td>
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<tr>
<td>mys</td>
<td>myospheriod</td>
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<tr>
<td>PCD</td>
<td>Programmed Cell Death</td>
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<tr>
<td>PINCH</td>
<td>Particularly Interesting New Cysteine and Histadine rich protein</td>
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<td>PIV</td>
<td>Particle image Velocimetry</td>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>PSI</td>
<td>plexin-semaphorin-integrin</td>
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<tr>
<td>PTB</td>
<td>phosphotyrosine binding</td>
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<tr>
<td>RIAM</td>
<td>Rap1-GTP Interacting Adaptor Molecule</td>
</tr>
<tr>
<td>RLC</td>
<td>Myosin regulatory light chains</td>
</tr>
<tr>
<td>ROCK</td>
<td>Rho-associated protein kinase</td>
</tr>
<tr>
<td>sqh</td>
<td>spagetti-squash</td>
</tr>
<tr>
<td>( t_{1/2} )</td>
<td>Half Time of Recovery</td>
</tr>
<tr>
<td>VBS</td>
<td>Vinculin Binding Sites</td>
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<tr>
<td>YFP</td>
<td>Yellow Fluorescent Protein</td>
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# List of Fly Gene Names and Vertebrate Homologues

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<tr>
<th>Fly Gene Name</th>
<th>Vertebrate Homologue</th>
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<td><em>Decapentaplegic</em></td>
<td>Bone Morphogenetic Protein</td>
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<td><em>myospheroid</em></td>
<td>Integrin Beta 1/3</td>
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<td><em>rhea</em></td>
<td>Talin</td>
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<tr>
<td><em>spaghetti-squash</em></td>
<td>Myosin II regulatory light chain</td>
</tr>
<tr>
<td><em>zipper</em></td>
<td>Myosin II heavy chain</td>
</tr>
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Last but not least, I would like to thank my flies. People like to laugh about them,
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have been possible without them.
CHAPTER ONE: Introduction

1.1 Morphogenesis

The study of developmental biology aims to unravel the biological processes by which life grows and develops. Animal development primarily occurs during embryogenesis, during which the body plan is established and the majority of cell differentiation occurs. The earliest studies of metazoan development focused primarily on descriptive analysis of conserved structural changes during development. Importantly, all animals derive from a basic set of body plans, which suggests the existence of basic rules of construction, with variety arising from subtle variation on a few conserved themes. However, led by the discovery of morphogens (Turing, 1990), modern developmental biology focuses on understanding spatiotemporal gene expression and how morphogen gradients give rise to complex patterning during embryogenesis.

Recently, researchers have begun to fuse the two areas together, creating a more global model incorporating large-scale tissue transformations into the modern molecular picture of development (Wozniak and Chen, 2009). In this comprehensive view, stereotypical structural rearrangements arise from a precisely orchestrated series of cell fate specification events and cellular movements (Walck-Shannon and Hardin, 2014). Current studies of morphogenesis aim to understand how cellular-level molecular changes can orchestrate large-scale tissue rearrangements, and thus sculpt the embryo’s three-dimensional shape.
Morphogenesis enables the proper spatial distribution of cells within embryos and organ development. As tissues develop they undergo a diverse set of changes, such as bending, lengthening, narrowing, branching and folding (Munjal and Lecuit, 2014). However, only a limited range of cellular behaviours such as division, death, growth, migration and shape changes is sufficient to drive these highly variable changes in tissue shape (Lecuit and Lenne, 2007). It is the coordination of these individual cellular processes across tissues which gives rise the genetically-programmed final form of an organism at the end of embryogenesis.

Ultimately, there are three functional modules that contribute to morphogenesis (Mateus et al., 2009). First, the cytoskeletal machinery is the physical force that generates the structure necessary to undergo morphogenetic processes (Section 1.2). Secondly, adhesion, or linkage between cell to one another and to the extracellular matrix, provides links and anchors for the cytoskeleton as well as coordinating changes in cells across tissues. These two first elements together give rise to stereotypical cell behaviours, the third module, which are a crucial element generating the large scale tissue wide changes driving morphogenesis (Section 1.5). The relationship between these modules is illustrated in Figure 1.1. Ultimately, generating a more holistic view of morphogenesis, in which both large-scale tissue wide behaviours, and molecular contributions of proteins at the cellular level are considered will provide insight into development on a systems level. This thesis aims to contribute to this modern view of development through consideration of the role of adhesion upon a specific force-dependent morphogenetic event, dorsal closure (DC) (Chapter 3), as well as consider the consequences of the finely-tuned regulation of adhesion upon the stabilization of muscle architecture (Chapter 2).
1.2 Dorsal Closure (DC)

A few developmental processes during embryogenesis in model organisms have emerged as excellent models of morphogenesis. These models illustrate the complex interplay of different cell behaviours that result in the formation of three-dimensional shapes as well as the ongoing role for actomyosin contractility and cell adhesion in morphogenesis. There are many diverse models of morphogenesis that have emerged over time. This thesis focuses on one example during fly development, DC. Importantly, DC shares many characteristics with neural tube closure, highlighting the broad conservation of these processes and the relevance of DC to vertebrate models of development.

1.2.1 Overview of DC

DC is a morphogenetic process that occurs about 10 hours into fly development and shares characteristics with vertebrate neural tube closure and wound healing (Harden, 2002; Wood et al., 2002). Importantly, loss of integrin mediated adhesion results in severe defects in DC. During DC two lateral epithelial sheets advance over an extra-embryonic tissue known as the amnioserosa (AS) to seal a naturally occurring eye-shaped hole in the dorsal epidermis of the fly embryo (Figure 1.2A). Both the lateral epidermis and the AS are under tension and contribute to DC (Kiehart et al., 2000). A number of key proteins contributing to DC, including integrins, have been identified genetically (Harris et al., 2009; Narasimha and Brown, 2004) (Gorfinkiel et al., 2011; Jacinto et al.,
2002). Importantly, myosin has shown to be required for the cellular behaviours driving DC (Franke et al., 2005).

The many forces that contribute to DC have been studied extensively (Hutson et al., 2003; Kiehart et al., 2000; Solon et al., 2009; Toyama et al., 2008). DC thus has emerged as an excellent in vivo model to study the contributions of various proteins to regulated tissue biomechanics, due to the experimental accessibility of Drosophila, the clear role of biomechanics in this process and well-developed understanding of the forces contributing to it.

1.2.2 Forces driving DC

There are multiple redundant forces that contribute to DC (Jacinto et al., 2002). Importantly, both the lateral epidermis and the AS contribute to DC. Specifically, the lateral epidermis provides resistive tension against the strong closing force generated by the AS, and cells in the epidermis elongate in the dorso-ventral direction. Thus throughout DC, the lateral epidermis provides a resistive force, impeding closure (Wells et al., 2014). The AS progressively reduces its surface area through a variety of mechanisms that allow it to generate tension and provide strong closing force. Additionally, both of these tissues contribute to a supracellular actomyosin cable composed of myosin and actin that forms at the leading edge of the epidermis, and is thought to exert a contractile force to draw the hole closed (Figure 1.2B) (Hutson et al., 2003; Rodriguez-Diaz et al., 2008).
The AS generates closing force in multiple ways. First, cells within the AS itself generate tension across the AS through pulsatile apical cell constrictions and apoptosis-induced decreases in tissue area (Franke et al., 2005; Solon et al., 2009; Toyama et al., 2008). Apical constrictions within the AS are an intrinsic property of AS cells and are coordinated across the tissue, and exhibit temporal changes in amplitude and period of their pulses as DC progresses (Solon et al., 2009). Specifically, the pulses become dampened as closure progresses. Recently, an additional mechanism for producing tension in the AS has been proposed (Saias et al., 2015). Saias and colleagues suggested that cell volume shrinkage can also contribute to tissue contraction.

The final force contributing to DC occurs in the late stages of closure when the epidermal sheets are in close proximity to each other, the cells in the epidermis generate microtubule based protrusions that ‘zipper’ the tissue closed (Jankovics and Brunner, 2006; Peralta et al., 2007). All four of these forces contribute to wild-type closure, however, laser ablation and biomechanical approaches have demonstrated that none of these forces are absolutely required for DC (Figure 1.2) (Kiehart et al., 2000). However, it has since been shown that contraction generated by the AS in the absence of purse-string tension is sufficient for drive DC (Wells et al., 2014). Ultimately, the complex interplay of different cell behaviours including: apical cell oscillations, cell apoptosis, and actomyosin purse strings demonstrate that DC is an excellent model in which to study the forces that drive morphogenesis and the cellular events that regulate them.
1.2.3 Signalling events contributing to DC

Two major signalling pathways are known to contribute to DC: the stress response pathway Jun-N-terminal kinase pathway (JNK) lies upstream of the bone morphogenetic protein homologue Decapentaplegic (DPP). Embryos mutant for components of either of this two pathways have defective DC and exhibit open dorsal hole phenotypes (Ducuing et al., 2015). The connection between these two pathways during DC has recently emerged from work by Ducuing and colleagues, who find that DPP and JNK function together in a feed forward loop, in which JNK provides patterning information that is spatially filtered by DPP.

1.3 Actomyosin Contractility in Morphogenesis

In order for the dramatic rearrangements and cell shape changes involved in morphogenesis to occur, cells must be able to both generate and oppose forces as well as connect this tension across tissues via cellular adhesion. The primary force-generating machinery within the cell is built up of actomyosin networks. Actin and myosin form an interconnected network at a band localized to the cell cortex (Rauzi et al., 2008). Contractility in cells is generated when myosin motors pull on anti-parallel actin filaments; when these filaments are linked to cellular structures, tension across cells can be generated. Cells can regulate the production of force in a number of ways: by modulating the behaviour of myosin motors, altering the assembly and architecture of the actin array, and specifying the localization of actomyosin networks. In order for morphogenesis to occur, contractility of the actomyosin network must be tightly controlled in space and time.
1.3.1 Myosin

The myosin superfamily of proteins are motor proteins that hydrolyze adenosine triphosphate (ATP) to move along actin filaments. The N-terminal head domain is found all myosin proteins, and is a conserved catalytic domain. The head contains the actin binding sites and ATP-hydrolysis sites and is therefore the key site of force production. The diversity of the myosin family is primarily found in the variety of domains in the C-terminal tails (Hartman and Spudich, 2012). Most myosins exist as monomers, but non-muscle myosin II (Myo II), considered a conventional myosin and of particular interest to this study, exists as a hexamer (Hartman and Spudich, 2012) (Lecuit et al., 2011). These hexamers on their own are incapable of much activity, but they then assemble into bipolar filaments which can undergo dynamic assembly and disassembly and perform a range of functions in the cell, including generating cortical tension, mediating cytokinesis and driving cell shape changes.

The Myo II hexamer is composed of three subunits two heavy chains, two myosin regulatory light chains (RLC) and two essential light chains (ELC). Myo II is primarily regulated by phosphorylation on the RLC on two highly conserved residues (T18 and S19 in mammals, T20 and S21 in Drosophila) (Munjal and Lecuit, 2014). These phosphorylation sites are the target of numerous kinases, including Rho associated protein kinase (ROCK), myotonia dystrophy related Cdc-42 binding kinase and myosin light chain kinase (MLCK) (Martin et al., 2009). MyoII activity is therefore highly regulated, both by phosphorylation, but also by conformational changes. In its native state, Myo II adopts an inactive closed formation. MRLC phosphorylation promotes the unfolding of the homodimer and allows for the assembly of myosin microfilaments.
(Lecuit et al., 2011). *Drosophila* is an excellent model to study myosin regulation, as it has a single gene for each of the 3 subunits of Myo II: *zipper* encodes the heavy chain, *spaghetti-squash (sqh)* encodes the RLC, and the ELC is encoded by *essential light chain-cytoplasmic* (Franke et al., 2006).

### 1.3.2 Actin

In order for myosin to function, it needs to be associated with anti-parallel actin filaments. The organization of actin itself also has profound effects on morphogenesis. Cells generally have a pool of actin monomers that can be assembled into actin filaments. Actin networks can either unbranched, mediated by formin family of proteins, or assemble into dendritic networks, promoted by Arp2/3-mediated polymerization (Levayer and Lecuit, 2012). These two different types of networks have different consequences for the actin network architecture and mechanical properties. Unbranched networks can be arranged in arrays, and enhance the recruitment of Myo II and therefore contractility. Furthermore, unbranched filament can be bundled together, mediated by actin binding proteins such as α-actinin and fimbrin, in which parallel actin fibres are assembled together such that they can effectively act as an elastic rod, generating robust structures that can mediate cell protrusions such as filopodia. Since dendritic networks are crosslinked, they can be considered to be stiffer and require breaking down in order to modulate contractility (Kasza and Zallen, 2011). In reality, the two populations of actin are often intermixed, and therefore the role of each is not always clear, but it is nevertheless clear that actin organization plays a key role in the modulation of cellular contractility (Lecuit et al., 2011; Levayer and Lecuit, 2012).
1.3.3 Actomyosin dynamics

Recent advances in live-imaging techniques have greatly improved the ability for visualization of cell behaviours in vivo. These analyses have revealed that actin and myosin form a remarkably dynamic network, conserved across species and cell types. There are two main behaviours observed: flows and pulses. Flows occur when deformations allow actin filaments to slide and undergo remodelling and altering of their cross-linker architecture. This is will cause the energy from the deformation to dissipate in a fluid like manner, which can be described as a flow (Levayer and Lecuit, 2012). Flows have been show to be generated by gradients of contractility (Mayer et al., 2010) as well as by asymmetric coupling to E-cadherin clusters (Levayer and Lecuit, 2013). Pulses are characterized by rapid fluctuations of actomyosin intensities in the order of ten of seconds (Munjal and Lecuit, 2014). These rapid changes are cause by cycles of recruitment and coalescence of Myo II to generate pulses that cause rapid cell deformations before they are disassembled (Martin et al., 2009). These pulses often occur between periods of stabilization which results in an overall irreversible shape change process (Mason and Martin, 2011).

1.4 Adhesion in Morphogenesis

In order for the tension generated by pulling on actin networks to affect the tissue shape, and therefore result in cell behaviours, actomyosin networks need to be physically linked to the cellular structures, and/or its external environment. This coupling is achieved by adhesion complexes. Adhesion is therefore a key component of
morphogenesis, as it allows changes at the individual cell level to be coordinated across tissues.

Cells can adhere via many different adhesion molecules, but there are two primary connections that they make: cell-cell adhesions, primarily via the cadherin family of proteins, and connections between cells and the extracellular matrix (ECM), primarily mediated by integrins. These two classes of adhesion receptors form connections to the actin cytoskeleton, and contribute to its regulation (Roper, 2015). Cadherins and integrins are equally crucial to morphogenesis, however since integrins are the main focus of this thesis, they will be discussed in more detail later in this introduction (section 1.6). Importantly, both sets of adhesion molecules are crucial to DC.

1.4.1 Cadherins

Cadherins derive their name from their dependency on calcium ions for their function. There are many different types of cadherins that form a variety of cell-cell adhesive structures in many contexts and thus cadherins have many diverse roles in development. A key family of cadherins are classic cadherins, called classic because they were the first cadherins identified (Harris and Tepass, 2010). Classic cadherins are type I transmembrane proteins, which form the base of adherens-junctions (AJs), a key component of epithelia, which are the main site of adhesive cell-cell junctions. Importantly, in Drosophila there are only 3 classic cadherins (1 E-cadherin, and two N-cadherins)(Bulgakova et al., 2012). AJ’s are primarily composed of epithelial cadherin (E-cadherin). E-cadherin has an extracellular domain that participates in homophilic interactions in trans between molecules on neighbouring cells. E-cadherins then span the
membrane, and bind cortical actin and regulatory factors that mediate its functionality on their cytoplasmic side (Roper, 2015). The cytoplasmic domains of classic cadherins are highly conserved and a key family of proteins making links between cadherin cytoplasmic tails and actin and microtubules are the catenin family of proteins. While specific interactions between the cadherin-catenin complex and actin are mostly likely context specific, it is clear that catenin’s are central to the connections between cadherins and actin.

1.5 Cell Behaviours Driving Morphogenesis

There are a few conserved cellular behaviours that are frequently utilized in the establishment of three-dimensional structures during morphogenesis. Examples of these mechanisms include: programmed cell death (PCD), epithelial to mesenchyme transition oriented cell divisions, cell intercalation, contractile actomyosin ring assembly, and apical cell constrictions. Of these cell behaviours, PCD, contractile actomyosin rings, and apical cell behaviours are all crucial to DC, and will therefore be discussed in depth.

1.5.1 Cell divisions

As organisms grow and develop, cell division is controlled to determine the number of cells and overall size of a multicellular organism (Morin and Bellaiche, 2011). The control of cell division in time and in space can affect the shape and growth of tissues. The orientation of cell divisions is determined by the alignment of the mitotic spindle which can be controlled by multiple mechanisms including: cell shape anisotropy,
biochemical signals from the surrounding environment and externally applied forces (Campinho and Heisenberg, 2013). An excellent example of the role of coordinated cell divisions occurs in the Drosophila wing disc epithelium (Mao et al., 2011). Here, Mao and colleagues demonstrated that symmetric cell divisions within the wing disc align preferentially with the proximal-distal axis, thus elongating the organ specifically along this axis. Interestingly, they provide evidence that an anisotropic landscape of tension across the tissue is responsible for defining the orientation of these cell divisions. This is illustrated in Figure 1.3B, in which different rates of cell divisions result in the accumulation of tension along the proximal distal axis (tension anisotropy), resulting in changes in the orientation of the mitotic spindle.

1.5.2 Cell intercalation

Cell intercalation involves highly organized exchanges between neighbouring cells without an overall change in cell number (Walck-Shannon and Hardin, 2014). Intercalation events can occur throughout development, and are involved in processes ranging from early development, when the germ layers are organized in gastrulation, to convergent extension in later embryogenesis, which underlies the elongation of epithelial tubes (Walck-Shannon and Hardin, 2014). Convergent extension is a process in which a tissue narrows along one axis, and lengthens along the other, thus conserving cell number while achieving drastic changes in overall shape (Figure 1.3 C). An elegant example of cell intercalation driving convergent extension occurs during germband extension in Drosophila. During this process cells intercalate between their dorsal and ventral neighbours thus lengthening the anterior posterior body axis (Irvine and Wieschaus,
1994). This precise directional movement has been shown to be dependent on irreversible and planar polarized remodelling of epithelial cell junctions, and by the flow of medial actomyosin pulses to these junctions (Blankenship et al., 2006) (Rauzi et al., 2010).

1.5.3 Programmed cell death

PCD is an integral component of animal development. PCD was first observed as apoptosis in cells undergoing cell death that exhibited distinct morphological changes, shrinking, condensing and maintaining their membrane integrity during animal development or tissue homeostasis (Kerr et al., 1972). By contrast, cells involved in pathological cell death underwent swelling and rupturing, characterized as cell necrosis and typically observed as acute lesions (Jacobson et al., 1997). The definition of PCD has since become more nuanced as the pathways initiating it were discovered in C. elegans, and now PCD is defined as any cell utilizing an intracellular death program to die.

PCD is essential to many developmental processes, including sculpting and deleting structures, regulating cell numbers and the elimination of unnecessary cells. The classic model of PCD is digit individualization in vertebrates (Suzanne and Steller, 2013). In this process, interdigital webs are eliminated utilizing the apoptotic machinery, to gradually “sculpt” the tissue into the correct form (Fuchs and Steller, 2011; Suzanne and Steller, 2013). This is supported by the observation that in organisms with webbed digits, scant cell death is observed in the interdigit region (Suzanne and Steller, 2013).
Apoptosis through PCD is a crucial force driving DC. During DC approximately 10% of cells constrict their apical surface and delaminate out of the AS (Kiehart et al., 2000). Later these cells were found to demonstrate the hallmarks of apoptosis, including blebbing and cell fragmentation (Toyama et al., 2008). Furthermore, when the ability to undergo apoptosis in the AS was either induced or inhibited using the UAS/GAL4 system, this had significant effects on the ability of the embryo to undergo DC, suggesting that ‘apoptotic force’ plays a key role in driving DC.

1.5.4 Contractile actomyosin rings

Although classically a key component of cytokinesis, contractile actomyosin rings can encompass multiple cells, generating ‘supracellular’ cables (Figure 1.2E). This phenomenon has been reported in a variety of developmental contexts, including Drosophila DC (Solon et al., 2009), zebrafish gastrulation (Behrndt et al., 2012), cell sorting in the Drosophila wing disc (Landsberg et al., 2009) and embryonic wound repair (Fernandez-Gonzalez and Zallen, 2013). In these contexts is it hypothesized that these supracellular actomyosin cables may act to coordinate contractility across many cells. Importantly, there remains debate over the role of these purse strings, especially in DC (Wells et al., 2014). However, it is clear that during late embryonic wound repair in the epidermis of Drosophila, a contractile purse string composed of actin and myosin is formed, which then contracts to coordinate the inward movement of cells adjacent to the wound (Fernandez-Gonzalez and Zallen, 2013).
1.4.5 Apical constrictions

The constriction of the apical surface of the cell has been hypothesized since 1902 to contribute to the bending of a sheet of cells (Sawyer et al., 2010). Depending on the physiological context and local topography, apically constricting cells can have different consequences for tissue geometry. Apical constriction can therefore facilitate tissue bending, ingestion, or contraction during morphogenesis (Martin and Goldstein, 2014). Apical constriction thus underlies a diverse array of developmental processes, ranging from gastrulation in *Drosophila* (Askari et al., 2009), to DC, and including neural tube closure in vertebrates. During DC apical constrictions within the AS are coordinated across the tissue to generate tension in the AS, a crucial force driving DC. Another dramatic example of the impact of apical constrictions is *Drosophila* mesoderm invagination. In this process, beginning at gastrulation, the apical constriction of the ventral cells facilitates the formation of a furrow along the ventral side of the embryo. These cells fold entirely out of plane and into the interior of the embryo, thus generating the presumptive mesoderm, all in approximately 5 minutes (Martin et al., 2009).

It is the study of *Drosophila* gastrulation that first suggested that despite the variety of events driven by apical constrictions, there might be a universal force generating mechanism underlying apical constriction for all cell types (Martin and Goldstein, 2014) (Martin et al., 2009). Indeed, common machinery involving actin, the motor protein myosin and cell-cell adhesion components have been identified (Martin and Goldstein, 2014). Cadherin based cell-cell adhesion complexes have been shown to be crucial to apical constrictions, and have been hypothesized to act as a molecular clutch, connecting actomyosin generated tension across cells (Roh-Johnson et al., 2012).
However, although these components are essential to apical constrictions, their organization and dynamics are variable, and it is unclear if a central mechanism exists (Martin and Goldstein, 2014).

Interestingly, in both Drosophila mesoderm invagination and DC, apical constriction proceeds in a pulsatile manner – this behaviour has been called ratcheting (Blanchard et al., 2010; Martin et al., 2009). Here, cells undergo apical area fluctuations of gradually decreasing amplitude. During these cycles, there are periods of rapid decrease in apical area, followed by periods of stabilization in which the apical area relaxes slightly or does not significantly change (Figure 1.2D). Thus, in this case, apical constrictions are occurring in a stepwise manner. Importantly, these fluctuations in area dynamics have been shown to correlate with myosin dynamics (Martin et al., 2009).

1.6 Integrin Mediated Adhesion

Integrins are heterodimeric transmembrane type I adhesion receptors composed of an alpha (α) and beta (β) subunit. Integrins and their associated proteins provide a direct link between the ECM and intracellular actin cytoskeleton through membrane associated macromolecular complexes known as focal adhesions (FAs). FAs can contain hundreds of molecules but are integrin based (Case and Waterman, 2015). Integrins bi-directionally relay signals across the membrane as well as act as physical anchors for the cell. As such, integrins are involved in many fundamental biological processes such as cell adhesion, migration, survival and differentiation (Iwamoto and Calderwood, 2015).
Humans have 18α and 8β subunits that can combine to form 24 unique heterodimers creating diversity in integrin ligand specificity, tissue distribution, binding properties and therefore varied biological functions. *Drosophila* have 5α and 2 β subunits. Importantly, integrins and their associated proteins are highly conserved across metazoa (Maartens and Brown, 2015a). Out of the two β subunits only the βPS subunit is broadly expressed in *Drosophila*, while the other subunit βν is very tissue specific, and is only highly expressed in the midgut suggesting that it plays a tissue specific role during development (*Yee and Hynes, 1993*) (Maartens and Brown, 2015b). This is convenient for genetic analyses because removal of the βPS subunit essentially eliminates integrin function in the fly, as integrins are obligate heterodimers. Ultimately, the broad conservation of integrins across species, their involvement in many different biological processes and their diversity of expression suggests that integrins need to be tightly regulated in order to achieve their diverse biological functions.

1.6.1 Integrin activation

The ligand binding activity of integrins is known to be regulated by conformational changes (Askari et al., 2009; Frelinger et al., 1990). Integrins adopt a bent (low ligand affinity) state, which can be changed into an active, extended (high ligand affinity) state. The nature of the precise ectodomain rearrangements that leads to integrin activation remains controversial, but there is consensus that activation involves the separation of the alpha and beta cytoplasmic and transmembrane domains, driven by the movement of the β integrin hybrid domain (Iwamoto and Calderwood, 2015).
Importantly, integrin activation is a key way to regulate integrin activity and function. A protein involved in integrin activation is talin. Talin is a large cytoplasmic linker protein that binds integrin cytoplasmic tails and is key to integrin function. Specifically, upon binding of membrane-bound talin via a phosphotyrosine-binding (PTB) like domain in the 4.1/Ezrin/Radixin/Moesin (FERM) domain of the talin head to a conserved NPLY motif in the beta integrin tail, the $\beta$ subunit transmembrane domain becomes reoriented, which leads to integrin activation (Iwamoto and Calderwood, 2015). Another protein implicated in integrin activation is kindlin, which bind the membrane distal NPxY motif of $\beta$ tails and is known to cooperate with talin (Calderwood et al., 2013).

1.6.2 Integrin clustering

While integrin activation increases the affinity of individual receptors for their ECM ligands, integrin clustering can increase the avidity of integrins interaction with the ECM by forming multiple localized bonds to the substrate to create stronger, more mature adhesions. The molecular basis of clustering has yet to be elucidated, but multivalent ECM ligands have been implicated in integrin clustering, as has the cell glycocalyx (the exterior cell surface layer above the plasma membrane composed of carbohydrate moieties glycoproteins and polysaccharides) (Kim et al., 2004) (Paszek et al., 2014). Integrin clustering is coincident with the strengthening and maturation of integrin adhesions. Maturation of integrin adhesion is known to involve the recruitment of a complex of proteins known as the integrin adhesome. Assorted proteins in the integrin adhesome have been implicated in integrin activation, including talin, kindlin and $\alpha$-actinin (Iwamoto and Calderwood, 2015; Saltel et al., 2009). Importantly, these
proteins are also implicated in integrin conformational changes, thus suggesting that integrin activation and clustering are closely linked, and making distinguishing separate roles for integrin activation and clustering challenging.

1.6.3 Integrin structure

The structure of integrins has been well characterized, through functional and in vitro structural studies. Importantly, this understanding is informed by determination of the crystal structure of an integrin dimer, first solved in 2001 (Xiong et al., 2001). The shape of integrins is broadly described as a large “head” resting on two “legs” composed of the alpha and beta subunits. The overall structure takes a “bent” formation, placing the ligand-binding site near the surface of the membrane (Humphries et al., 2003). The alpha and beta subunits associate non-covalently, and each are composed of functional domains separated by flexible linkers. Each subunit contains the majority of its structure extracellularly, but both subunits also contain membrane spanning helices, and a short cytoplasmic tail. Although the size is variable, alpha subunits are usually around 1000 amino acids, and beta are around 750 (Campbell and Humphries, 2011).

The “head” domain of beta integrin contains the ligand-binding βA domain, which is then connected to hybrid domain, followed by the plexin-semaphorin-integrin (PSI) domain (Askari et al., 2009). The beta leg is then composed of four EGF repeats and the β tail domain. The structure of the beta cytoplasmic tail is unclear, but studies suggest transient and flexible structures (Campbell and Humphries, 2011). However, the
beta tails are key to integrin function. Intracellularly, beta integrin tails bind a diverse array of proteins known at the integrin adhesion complex (IAC). Another important domain contained the beta integrin is the hybrid domain. This domain is important because when integrin binds to ligand the hybrid domain is known to move outward, representing the key step in switching between active and inactive integrin (Askari et al., 2009)

1.7 Talin

Talin is a large (750 kD) cytoplasmic scaffolding adaptor protein that forms a key component of FA’s. The N-terminal head domain of talin binds directly to cytoplasmic β integrin tails and therefore is localized at the membrane. Importantly, talin can also directly bind actin, and therefore acts as a direct linker between the integrins at the ECM and the actin cytoskeleton. There are numerous actin binding sites (ABS) in talin (Franco-Cea et al., 2010), but talin primarily binds actin in its tail region, which has been shown to be localized approximately 30 nm above the plasma membrane (Case and Waterman, 2015; Kanchanawong et al., 2010). Talin is conserved throughout metazoans; although vertebrates have two homologues of the gene (tln1 and tln2) while Drosophila only have one (rhea). As talin is essential for integrin function and signalling and it’s function is tightly regulated, it represents an ideal target to study integrin regulation during development.
1.7.1 Talin structure

As talin acts as a molecular scaffold it contains many protein-protein interacting domains. Understanding the structure of these domains is crucial to elucidating the role of talin as an integrin regulator, understanding how it couples integrin to the cytoskeleton and determining key properties of the protein itself. Although the crystal structure of full-length talin has not been solved, the 18 domains that comprise talin have each been determined, allowing a model for full-length talin to be formed (Calderwood et al., 2013). These domains can be broken into two main components: the talin head (aa 1-400) and the talin rod (aa 487-2529), which are connected by a flexible unstructured linker (Calderwood et al., 2013).

The N terminal globular head domain of talin is approximately 50 kDa. It contains an atypical FERM domain, composed of four (rather than the typical 3) lobular subdomains (F0, F1, F2 and F3). The F3 lobe contains a PTB-like domain that is known to bind the proximal NPxY motif b-integrin tails (integrin binding site 1 or IBS-1) as well as bind to several kinases (PtdInsP kinase Ig, Focal adhesion kinase, (FAK)) (Critchley, 2009). Furthermore, the F3 domain is known to be required for integrin activation (Calderwood et al., 2002) although it is not sufficient, and interactions between the talin head and the plasma membrane are also critical. Specifically, basic residues along the membrane proximal surface of the talin head allow the head to positioned optimally along the plasma membrane such that it can mediate the re-orientation of the β-integrin transmembrane domain and lead to integrin activation (Critchley, 2009).

The talin rod is composed of 62 amphipathic α-helices that are organized into 13 bundles each composed of 3-4 helices (R1-R13). The rod domain contains many protein
binding sites such as; vinculin binding sites (VBS), actin binding sites (ABS), a second integrin binding site (IBS-2 (Ellis et al., 2011)), binding sites for Rap1AGTPase effector RIAM (Rap1-GTP Interacting Adaptor Molecule), as well as sites for other assorted interactions. Furthermore, R9 of the rod domain contains a site that the talin F3 lobe can bind to, allowing talin to auto inhibit itself to regulate its activity. (Ellis et al., 2013) (Calderwood et al., 2013). Importantly, the rod is also able to regulate talin activity by responding to mechanical force by unfolding and exposing cryptic vinculin binding domains (del Rio et al., 2009). Therefore the talin rod is an important protein-binding scaffold and also a site of talin regulation.

1.8 The Integrin Adhesion Complex during Development

Given the role of integrins in many diverse biological functions, it is unsurprising that loss of integrin-mediated adhesion has been shown to be essential for embryonic development in many contexts. Importantly, mutations in some or all of the alpha and beta subunits have been shown to be lethal in mice, flies, zebrafish and worms (Maartens and Brown, 2015a). This highly conserved role of integrins in metazoan development, as well as the diverse phenotypes that loss of integrin can result in demonstrates the many roles that integrin adhesion plays during development. Furthermore, this underscores the importance of integrin regulation in morphogenesis.

During Drosophila development, the βPS subunit is essential for a diverse list of processes and flies lacking βPS exhibit a range of phenotypes. These phenotypes reflect loss of integrin in dynamic short-term adhesions contributing to morphogenesis, such as
failures in DC. Furthermore defects are also observed in processes relying on long-term stable adhesions such tissue maintenance such as during muscle development, resulting in rounded up, detached muscles. Thus, Drosophila embryogenesis represents an ideal system in which to study the functional regulation of integrin adhesion during development. Our lab utilizes a structure-function approach to gain insight into the functional regulation of integrin adhesion during development. Earlier work from our lab has found the different structural domains in integrin contribute to its different roles during development (Pines et al., 2011) and that mechanical force stabilizes integrin adhesion in vivo (Pines et al., 2012).

Importantly, talin plays a key role in regulating integrin function during development. In Drosophila loss of talin phenocopies loss of integrin, demonstrating the key role of talin in regulating integrin function in Drosophila. Our lab has focused on using a structure-function approach to elucidate the role of talin in regulating integrin adhesion during Drosophila development, which this work intends to expand upon. We find that the different integrin binding sites in talin have specific roles in Drosophila development. Specifically, IBS-1 is required for reinforcing attachment between integrins and the ECM, and IBS-2 for mediating connections between integrin and the IAC (Ellis et al., 2011). Furthermore, recent studies from our lab have demonstrated the key role of talin autoinhibition in modulating the stability of cell-ECM adhesion, which has serious consequences for DC, resulting in delays (Ellis et al., 2013).
1.9 Aims and Scope of This Thesis

The aim of this thesis is to examine the mechanisms by which cell-ECM adhesion is regulated during morphogenesis. Much is known about the structural regulation of integrin, and its functional role during morphogenesis. Despite this understanding, work is ongoing to understand the \textit{in vivo} regulation of integrin during morphogenesis. This thesis attempts to answer one aspect of this question by investigating the role of talin head in mediating \textit{Drosophila} integrin function and considering the role of talin in mediating integrin avidity versus affinity (Chapter One). We hypothesize that the talin head plays a distinct role in integrin clustering during morphogenesis.

Furthermore, the contribution of integrin to stereotypical morphogenetic events during \textit{Drosophila} development, especially DC, has been well established. \textit{Drosophila} development is therefore an ideal system in which to study the role of integrin in contributing the specific cell behaviours that drive morphogenesis. I therefore explored the role of basal integrin in apical cell oscillations during DC (Chapter Two). Here, I hypothesized that integrin adhesion plays a key role in coordinating actomyosin dynamics within the AS during DC. Overall, my studies aim to investigate the regulation of the diverse functions that integrin mediated adhesion can achieve during development.
1.10: Chapter 1 Figures

Figure 1.1 Three modules contributing to morphogenesis.
Figure 1.2 Dorsal closure. During DC two encroaching lateral epithelial sheets (shown in blue) cover over an extra-embryonic tissue known as the AS (shown in yellow) to seal a dorsal hole in the developing fly (A). Many redundant forces contribute to this process, including lateral epidermis resistive forces (dark blue), tension in a supracellular actomyosin purse string (green) and tension within the AS (red arrows) which is generated through apical cell racheting (teal) and apoptosis induced decrease in tissue area (yellow).
Figure 1.3 Cell behaviours driving morphogenesis. Schematic of the different cell behaviours driving morphogenesis, including PCD (A), cell divisions (B), cell intercalation (C), apical cell oscillations (D) and actomyosin purse string formation (E).
Figure 1.4 The domain structure of the integrin heterodimer. The major conserved domains of the integrin heterodimer (A) shown in the extended conformation. (B) Schematic of the integrin domain crucial to this study with the point mutations used highlighted.
CHAPTER TWO: The Talin Head Domain Reinforces Integrin-Mediated Adhesion by Promoting Adhesion Complex Stability and Clustering.

2.1 Chapter Two Introduction

Integrins are the main family of cell-ECM adhesion receptors and play many diverse roles during animal development. The strength and stability of integrin-based adhesion varies in response to the functional context: stable, long-lasting adhesion is used to preserve tissue architecture while short-term matrix attachment is used for dynamic processes such as cell migration during embryonic morphogenesis. Therefore the regulation of cell-ECM adhesion duration and strength is crucial to the ability of embryos to undergo robust development. Integrins can be regulated in many different ways: conformational specific changes altering their affinity for the ECM ligands (integrin activation/affinity), multiple integrin heterodimers clustering into discrete adhesive structures (integrin avidity), mechanical forces exerted on integrins themselves, and intracellular trafficking of the receptor to the cell surface.

However, the two main mechanisms by which strength and duration of integrin binding to the ECM is controlled is through integrin activation and integrin clustering. During integrin activation the integrin heterodimer undergoes conformational changes that allow it to change from a low affinity “bent” state into a high affinity extended state, thus increasing the strength of integrin binding to ECM ligands (Xiong et al., 2001). By contrast, integrin clustering increases the avidity of integrin-based adhesions by forming
an increased number of integrin bonds at a single site of adhesions to create a stronger adhesive contact. It is clear that both integrin activation and integrin clustering play essential roles in various cellular contexts. However, it is not known whether all cells where integrins are known to function use these two regulatory mechanisms. An intriguing possibility is that there are tissue-specific contexts that require a particular mode of regulating integrin, either clustering or activation. Furthermore, as these two processes are likely linked (Iwamoto and Calderwood, 2015), experimental separation of the role for integrin clustering versus integrin activation remains challenging and requires the ability to abrogate clustering and/or activation, in vivo, across tissues and compare the observed phenotypes.

The large cytoplasmic linker protein talin is a key component of focal adhesions and is central mediator of both integrin clustering and integrin activation making it an ideal target for the dissection of the different contributions of these two processes. Specifically, the N-terminal “head” domain of talin is clearly implicated in binding to, and then activating integrins, in a process known as ‘inside-out’ activation. (Calderwood et al., 1999). The talin head is composed of an atypical FERM domain comprised by four lobular subdomains (atypical F0 and F1,F2,F3). The F3 subdomain of the talin head binds directly to integrin cytoplasmic tails and is crucial to inside out activation. However the F3 domain of the talin head is not sufficient to drive integrin activation. Basic residues in the other lobes of the talin head are crucial to allow the talin head to be optimally positioned along the plasma membrane(Cluzel et al., 2005). It is this positioning in conjunction with talin F3-integrin interactions that allows the induction in a change of the angle of the β-integrin transmembrane domain characteristic of
activation to occur. By comparison, the molecular mechanism underlying clustering has yet to be clearly elucidated, although it is clear that talin does play a role (Iwamoto and Calderwood, 2015). However, Saltel and colleagues suggest that similar membrane-talin-integrin conformational changes and interactions likely underlie integrin clustering as well (Saltel et al., 2009).

Studies in *Drosophila melanogaster* have generated useful insight into the regulation of integrin function in vivo (Ellis et al., 2011; Franco-Cea et al., 2010; Pines et al., 2011; Tanentzapf and Brown, 2006; Yuan et al., 2010). This is because the smaller genome of flies is particularly amenable to transgenic and mutational structure/function analysis, and there is less genetic redundancy to contend with than in higher organisms. Also, there is an array of developmental processes during fly development that are integrin-dependent allowing for integrin function to be analyzed in diverse contexts. Interestingly, previous work has investigated the role of talin head mediated integrin activation in vivo. Specifically, when a mutation that abolishes the ability of the F2-F3 domain of the talin head to bind to integrin cytoplasmic tails (R367A), via Integrin Binding Site 1 (IBS-1) was introduced into fly talin, the phenotype was surprisingly mild (Tanentzapf and Brown, 2006). Importantly, this mutation did not specifically affect integrin activation, but more broadly binding between the talin head and integrins. The specific phenotype observed was primarily in prominent sites of integrin adhesions in the fly muscles, known as myotendious junctions (MTJs). MTJ’s are key sites of integrin adhesion in the fly, where integrins mediate stable attachment between muscles and the specialized tendon cells of the overlying epidermis. In IBS-1 mutants, the colocalization pattern of integrins and ECM ligands observed at MTJs was disrupted, suggesting that the
attachment between integrins and the ECM was weakened, and as a consequence muscle attachment was impaired causing the embryos to die at the onset of muscle contraction (Tanentzapf and Brown, 2006). One possible interpretation of these results was that there was a second integrin binding site within the talin molecule that could functionally compensate for loss of IBS-1. However, this was shown to be unlikely as a subsequent study of IBS-2, a second integrin binding site found in the talin rod, demonstrated that while this additional interaction is key for dynamic adhesive events, and linkage between integrins and the intracellular adhesion complex, it was not required to maintain association between integrins and the ECM. It was therefore suggested that integrin linkage to IBS-1/the talin head is crucial for stabilizing cell-ECM adhesion in *Drosophila*, while IBS-2 binding is key for the establishment of Cell-ECM adhesions (Ellis et al., 2011). However, because the mutation used in these prior studies impinged on F2-F3 binding we cannot ascribe the observed phenotypes to specific failures in integrin activation. Intriguingly, work using insect cell-culture argues that the canonical talin-head induced integrin activation does not occur in *Drosophila* (Helsten et al., 2008).

Overall, the existing body of data suggests the complete picture of the role of the talin head, and specifically of talin-head dependent integrin activation, during Drosophila embryogenesis has yet to be elucidated.

Here, we utilize a structure/function approach to investigate the role of the talin head domain in the context of the developing fly embryo. We use targeted mutations in talin that abolish talin head-mediated integrin activation while leaving all other functions of the talin head intact. We are thus able to confirm that canonical talin head-mediated inside-out activation is indeed not essential for fly embryogenesis. Importantly, we
identify a point mutation in the talin head that phenocopies complete deletion of the talin head, and interferes with the reinforcement of cell-ECM adhesion. A key feature of this mutation is that it not only disrupts talin head domain-mediated integrin activation but also impinges on integrin clustering. Biochemical analysis provides a mechanistic basis for the phenotype underlying the mutation, identifying an essential intramolecular interaction between the F2 and F3 subdomains of talin. Our results suggest that a major function of the talin head is to induce integrin receptor clustering, and to promote adhesion maturation. Moreover, we provide genetic evidence demonstrating that clustering is the primary mechanism by which integrin function is regulated in developing fly embryos.

2.2 Chapter Two Results

2.2.1 Integrin activation is not essential for fly embryogenesis

We sought to introduce a mutation into the talin head that disrupted its ability to activate integrins but not other aspects of its function. We relied on the extensive knowledge of talin structure generated by previous NMR and crystallographic analysis of the talin-head interaction with integrin in order to do this. Previous studies identified a mutation (Figure. L325R in talin1, L331R in talin2) in mammalian talin that specifically abrogates the integrin-activating function of talin, but does not substantially affect the ability of the talin head to bind to integrin (Wegener et al., 2007). When this mutation is introduced into the talin head, it blocks the conformational change in integrin that drives activation. The residue identified specifically attenuates the interaction between the talin
head and integrin at the membrane proximal region of the \( \beta \)-integrin cytoplasmic tail, while maintaining the interaction between the talin head and the distal regions of the \( \beta \)-integrin cytoplasmic tail (Wegener et al., 2007). We introduced this mutation into fly talin (L334R) to study its effects.

Our lab has previously developed and extensively utilized a protocol to assess the effect of point mutations in fly talin. This protocol relies on the dominant-female sterile germline clone technique (Chou and Perrimon, 1996); (see Materials and Methods) to remove all the endogenous talin from fly embryos. To replace the endogenous talin we used ubiquitously expressed full-length talin rescue constructs, either wild-type (WT) talinGFP, or talin point mutants. The WT talinGFP construct rescued the embryonic lethality associated with loss of talin (data not shown). Surprisingly, we found that talinGFP*L334R-rescued embryos were in some cases indistinguishable from WT talinGFP-rescued embryos; we observed many L334R-rescued embryos hatching to the larval stages. Talin-dependent morphogenetic movements germband retraction (GBR) and DC were not affected by the L334R mutation (Figure 2.1A,B, SJE). However, we observed that approximately 20% of late stage 17 embryos possessed a form of muscle detachment defect. Previous work on talin*R367A, a mutation that abrogates talin binding to integrin via its IBS-1 domain and consequently blocks activation, also revealed late muscle detachment defects (Ellis et al., 2011; Tanentzapf and Brown, 2006). However, the late muscle defects in talin*R367A mutant were stronger and more penetrant than those of the talinGFP*L334R-rescued embryos.

A possible explanation for the difference between the R367A and L334R mutation would be that the L334R mutation might not block integrin activation in flies.
To directly test this possibility, we used a cell culture based activation assay to confirm that the L334R mutation indeed blocked the ability of the talin head to activate integrins. As was previously shown (Helsten et al., 2008), fly talin robustly activates human integrins when expressed in cell culture. In comparison, we found that the L334R mutation in the talin head abrogated integrin activation (SJE).

Consistent with the mild phenotype observed in talinGFP*L334R-rescues, we found a number of subtle differences in sub-cellular localization and dynamics between WT and L334R mutant talin. First, recruitment of talinGFP*L334R to sites of integrin-mediated adhesion was slightly reduced compared to WT talinGFP (Figure. 2.1E). This effect was more pronounced in the presence of endogenous talin suggesting that the mutant protein is outcompeted by the untagged WT protein for integrin binding (Figure. 2.1F). Second, we found that the turnover of talinGFP*L334R was elevated compared to that of WT talinGFP (Figure. 2.1G), when we employed a Fluorescence Recovery After Photobleaching (FRAP) protocol that we developed to analyze the stability of components of the integrin adhesion complex in sites of Cell-ECM attachment in the fly muscle (Pines et al., 2012; Yuan et al., 2010). Taken together, these data suggest that talin head-mediated integrin-activation, or at least L334-dependent activation, is dispensable for most of fly embryogenesis but does play a small role late in development in stabilizing adhesion to maintain tissue architecture.
2.2.2 A putative Rap-1 binding site in the talin head is dispensable for embryogenesis

We sought to identify roles of the talin head beyond integrin binding and activation. To this end, using the same approach described above, we replaced endogenous talin with a ubiquitously expressed construct that contains a point mutation in a positively charged residue in the talin head (K17E), which has been shown in vitro to bind to Rap1 in a GTP dependent manner (Goult et al., 2010). Interestingly, in Dictyostelium Rap1 was shown to bind directly to talin, and this interaction was essential to cell-ECM adhesion during growth and multicellular development (Plak et al., 2016). However, in Drosophila this mutation did not result in a severe phenotype (Figure 2.1C) and was also indistinguishable from wild-type talin rescue (Figure 2.1A), similar to our observations of talin*L334R. Furthermore, talin*K17E rescue did not significantly affect talinGFP localization, in the absence or presence of endogenous talin (Figure 2.1H,I, respectively). Similar, however, to the L334R mutation, FRAP analysis did reveal that talin*K17E had higher turnover (Figure 2.1J). This suggests that Rap1 binding to the talin head is dispensable for embryogenesis, although it does confer a small effect on talin stability at MTJ’s.

2.2.3 The talin head is required for integrin-dependent morphogenesis and muscle attachment

We therefore sought to clarify the importance of the talin head to Drosophila development. We replaced endogenous talin with a ubiquitously expressed construct that
deletes the talin head (residues 1-448) but leaves the rest of talin intact: headless-talinGFP. Importantly, head deletion resulted in severe phenotypes resembling complete loss of talin (Figure. 2.1D). GBR and DC were severely disrupted, as was stable muscle attachment to the ECM (SJE). While the headless-talinGFP localized poorly in the presence of endogenous talin it exhibited robust localization in talin null embryos (Figure. 2.1K,L). Therefore, headless-talinGFP was able to retain functional interactions that supported recruitment to sites of adhesion. Nonetheless, FRAP analysis showed that headless-talinGFP was substantially less stable at sites of adhesion (Figure. 2.1M). In addition, the adhesion complex that is normally recruited to sites of adhesion by talin (Becam et al., 2005; Brown et al., 2002; Tanentzapf and Brown, 2006), was absent or severely reduced in headless-talinGFP rescue embryos (SJE). These results show that deletion of the head results in severe defects in recruitment and stabilization of the adhesion complex; consequently, loss of talin head function blocks talin-dependent morphogenetic events.

2.2.4 rhea\textsuperscript{17} encodes a missense mutation in the talin head, G340E, required for talin function

Thus far, we have shown that talin-head mediated integrin activation plays only a minor role in talin function during fly development and that despite this, the head domain has other essential functions in mediating integrin-based Cell-ECM adhesion. To uncover the mechanism underlying additional roles for the head domain of talin we turned to a previously isolated allele of talin, rhea\textsuperscript{17}. This allele encodes a talin protein containing a missense mutation in the talin head and importantly, produces a phenotype that is similar
to that observed when the talin head is deleted in full (Figure 2.1D, Figure 2.2 D-SJE). The *rhea*<sup>17</sup> allele was originally uncovered in a screen for dominant enhancers of a hypomorphic integrin allele (Becam et al., 2005; Brown et al., 2002). We sequenced the *rhea*<sup>17</sup> allele and found that it contains a mutation that replaces a conserved glycine, G340 (G331 in mammalian talin1, G334 in mammalian talin2), to a glutamate (G340E; Fig. 2A,B). Phenotypic analysis of embryos homozygous for this mutation (Figure. 2.2E,F SJE) showed that integrin-dependent morphogenetic processes GBR and DC as well as stable muscle attachment to the ECM were severely disrupted compared to heterozygous controls (Figure. 2.2E,F SJE). Furthermore, upon closer analysis of DC defects, we found that *rhea*<sup>17</sup> mutant embryos have delays in DC, similar to those observed for an auto-inhibition mutant (Figure 2.2H)(Ellis et al., 2013).

A possible explanation for the strong phenotype observed in *rhea*<sup>17</sup> mutant embryos was that the mutation compromises the stability of talin protein such that functional defects observed could arise from insufficient levels of talin protein. However, analysis of *rhea*<sup>17</sup> embryos revealed that the G340E mutation did not affect either the localization to or levels of, both integrin (Figure 2.2I, SJE) and full-length talin at sites of adhesion at MTJs (Figure. 2.2J, SJE). Furthermore, Western blot analysis did not reveal any detectable degradation products associated with the *rhea*<sup>17</sup> mutation (SJE). Finally, side-by-side analysis of embryos heterozygous for either the *rhea*<sup>17</sup> allele or the *rhea*<sup>79</sup> talin null allele demonstrated that there was about twice as much talin protein in the *rhea*<sup>17</sup>/+ embryos compared to *rhea*<sup>79</sup>/+. This result indicated that full-length talin protein product from the *rhea*<sup>17</sup> allele was expressed at levels comparable to the wild type
allele, demonstrating that the talin protein containing the G340E mutation is stable and sufficiently expressed.

2.2.5 The G340E mutation abrogates integrin activation.

Since the full-length G340E talin encoded by the rhea^{17} allele is able to localize to sites of adhesion we asked whether this mutation blocked the ability of the talin head to bind to and activate integrins. To this end, we again employed a flow cytometry-based αIIbβ3 integrin activation assay. This showed that that the G340E point mutation, much like the L334R mutation, blocked the ability of the talin head to activate integrins (BM, SJE).

2.2.6 The G340E mutation affects integrin clustering.

The phenotype observed in rhea^{17} embryos cannot be explained by a defect in integrin activation alone since our data demonstrates that blocking activation by itself does not give rise to a severe phenotype (Figure. 2.1). Therefore, we hypothesized that the underlying cause of the rhea^{17} phenotype is due to a defect in another function associated with talin: integrin clustering (Cluzel et al., 2005; Saltel et al., 2009). Integrin clustering in the fly can be assessed using a well-established in vivo assay in the context of the fly imaginal wing disc epithelium (Becam et al., 2005; Brown et al., 2002; Franco-Cea et al., 2010; Tanentzapf and Brown, 2006). In the imaginal wing disc integrins mediate adhesion between the epithelial layers and form discrete puncta that colocalize with other adhesion complex components including talin, on the basal surface of the
epithelium (Brown et al., 2002). In the absence of talin these clusters fail to form, indicating a role for talin in integrin clustering (Figure. SJE 2.3C; (Brown et al., 2002)). Interestingly, clonal patches of homozygous rhea\textsuperscript{17} mutant cells also failed to form integrin clusters (Figure. SJE 2.3D). In comparison, neither the R367A mutation, nor the L334R mutation, disrupted integrin clustering (Figure. SJE 2.3E-F; (Tanentzapf and Brown, 2006)). These results are in line with the hypothesis that the G340E mutation in rhea\textsuperscript{17} directly impinges on the ability of talin to cluster integrins.

If the G340E mutation impacts integrin clustering, we predicted that this would affect the recruitment of integrins to sites of adhesion. To test this idea we analyzed integrin recruitment to MTJs. Consistent with previous reports (Devenport et al., 2007), integrin recruitment to MTJs exhibited a substantial increase between embryonic stages 16 and 17 in control WT embryos (Figure. 2.3A), but in rhea\textsuperscript{17} embryos, this increase did not occur (Figure. 2.3B). While this result hints at a defect in integrin clustering, it only provides indirect support for this hypothesis. Therefore, to more directly test the role of clustering we utilized neomycin, a reported inhibitor of integrin clustering that works by sequestration of PI(4,5)P2 membrane phospholipids (Arbuzova et al., 2000; Cluzel et al., 2005; Laux et al., 2000). It was previously shown that neomycin treatment results in increased turnover of integrins at focal adhesions (Cluzel et al., 2005). FRAP analysis of MTJs revealed that the mobile fraction of integrin-YFP increased by over 45% at sites of adhesion in embryos treated with neomycin compared to control vehicle-treated embryos (Figure 2.3G PLC). The mobile fraction of integrin-YFP also increased over heterozygous controls in rhea\textsuperscript{17} mutant embryos (Figure. 2.3H). Overall, we present three
lines of evidence which taken together, support the idea that clustering is disrupted by the presence of the G340E mutation in talin.

**2.2.7 rhea\textsuperscript{17} mutant embryos display defects in adhesion complex reinforcement.**

Both the fact that the talin head was required for adhesion complex assembly and that the G340E mutation interfered with integrin clustering (Figure. 2.3), led us to hypothesize that the \textit{rhea}\textsuperscript{17} mutation might give rise to defective adhesion complex assembly and maintenance. Since MTJs grow and mature over several hours of embryonic development (stages 16-17), they serve as a useful system to study the maturation of integrin-mediated adhesions. In WT embryos talin is localized at MTJs as they form during stage 15 and then undergoes substantial enrichment between stages 16 and 17 as Cell-ECM adhesions are consolidated and reinforced (Figure. 2.4A,C,E). Recruitment of other adhesion complex components including PINCH (Figure. 2.4 F,H,J) and pFAK (Figure. 2.4 K,M,N) also occurred at stage 16 and was maintained through stage 17. In \textit{rhea}\textsuperscript{17} embryos, although talin is well recruited to MTJs by stage 16, its recruitment is not reinforced in stage 17 (Figure. 2.4 B,D,E ). Strikingly, other adhesion complex components such as PINCH (Figure. 2.4 G,I,J) and pFAK (Figure. 2.4 L,N,O) were also initially recruited to MTJs at stage 16 at levels comparable to WT, but by stage 17, the levels were drastically reduced. Intriguingly, we found that MTJs are longer in \textit{rhea}\textsuperscript{17} embryos compared to WT controls, further suggesting a failure in adhesion maturation and consolidation (Figure. 2.4P-R). Altogether, these data demonstrate that the G340E mutation in the talin head impinges on the ability to reinforce integrin-
mediated adhesions, consistent with a defect in adhesion maturation, leading to the breakdown of the Cell-ECM adhesion.

2.2.8 The G340E mutation disrupts the interface between subdomains F2 and F3 in the talin head

We have shown that talin protein in *rhea* mutants fails to cluster integrins and reinforce integrin-mediated adhesions. However, the data presented so far does not explain the mechanism by which this effect is mediated. When put into the context of the large body of knowledge that exists about the structure of the talin head, the nature of molecular lesion in *rhea* provides some intriguing hints about this mechanism. Specifically, the G340E mutation is expected to disrupt the coordinated movement of the talin F2 and F3 domains, which is essential for activation and clustering. Structural modeling of the talin head, using the solved crystal structure of the mouse talin head in complex with β1-integrin ([Anthis et al., 2009]; PDB number 3G9W), predicted that residue equivalent to G340 (G331) is located on the surface of F3 at its interface with F2 (Figure 2.5A, inset BG). In the WT talin head this glycine allows the close packing of these two sub-domains. However, substitution for a glutamate inserts a charged carboxyl group into this close gap disrupting the fixed orientation of the F2 and F3 domains, which should allow them to move independently of one another. Since it has been proposed that a tri-partite interaction between integrin, talin head, and the phospholipid bilayer is required to facilitate stable adhesion and to promote inside-out signaling, it is quite possible that disrupting the coordination of F2 and F3 would destabilize these interactions. Consistent with such an effect the G340E mutation rendered the talin head
domain proteolytically sensitive to cleavage of the F3 subdomain from the F0-F2 subdomains (BG). This in vitro evidence is in line with the assertion that F2 and F3 become structurally uncoupled from one another when G340 is mutated.

Structural analysis of the mammalian talin head has shown that the orientation of the F2-F3 domains is fixed and that this has important implications for the talin function, for example during inside-out activation (Anthis et al., 2009). It was shown previously that a cluster of basically charged residues in the F2 domain, the membrane orientation patch (MOP; BG Figure 2.5A), play a key role in integrin activation via electrostatic interactions with the plasma membrane. In order to achieve optimal interaction of the MOP with the phospholipid headgroups, the F2-F3 module needs to reorient relative to the membrane. Multiscale molecular dynamics simulations suggest that this reorientation results in a ~20° change in the tilt of the β-integrin transmembrane domain (Kalli et al., 2010) leading to integrin activation via dissociation of the α-integrin and β-integrin transmembrane domains. Therefore, if the effect of the G340E mutation on clustering is due to disruption in the process of tilting and dissociation of the α-integrin and β-integrin transmembrane domains than mutations that specifically disrupt this process should also impinge on clustering. We therefore tested three mutations that have been proposed to have such an effect: first the L334R mutation in talin which was suggested to block the change in tilt angle of the β-integrin transmembrane domain (Wegener et al., 2007). Second, we tested two mutations in integrin previously suggested to promote dissociation of the α-integrin and β-integrin transmembrane domains, D807R (D723R in β3 integrin; Hughes et al., 1996; Lu et al., 2001; Pines et al., 2011)) and G792N (G708N in β3 integrin;(Li et al., 2003; Pines et al., 2011)). We found that clustering was similar to
wildtype for all three mutations when we replaced endogenous talin or integrin with the mutant proteins (Figure. 2.3F, Figure. 2.5B-E). We therefore did not find evidence that supports the idea that the G340E mutation is defective in clustering due to interference with tilting and dissociation of the α-integrin and β-integrin transmembrane domains.

Another possibility is that the G340E mutation disrupts the ability of the F2-F3 domains to bind the plasma membrane, which effectively reduces the affinity of the interaction between the talin head and integrin (Anthis et al., 2009). To test if specific membrane interactions might play a role in talin recruitment to sites of integrin-mediated adhesion, we again used the drug neomycin. Neomycin sequesters PIP2, which is the predominant phospholipid type that the talin head interacts with at the plasma membrane (Anthis et al., 2009). Consistent with this idea, we found that with neomycin treatment the recruitment of talinGFP to MTJs in embryos was significantly reduced compared to controls (Figure. 2.5F, SJE). In comparison, we failed to see a reduction in recruitment with the G340E mutation suggesting that the phenotype arises via a different mechanism than simple disruption of talin head’s interaction with the plasma membrane. This result implies that F2-F3 coordination could be important for integrin clustering through a mechanism other than by ensuring membrane embedding of the talin head domain.

2.3 Chapter Two Discussion

It has been well-established in mammalian systems that modulating integrin affinity, via interactions with the talin head is a key regulatory mechanism for integrins (Calderwood et al., 2002; Haling et al., 2011; Tadokoro et al., 2003). Yet, previous work suggested that integrin activation via the talin head may not be a major player in the fly
(Ellis et al., 2011; Helsten et al., 2008; Tanentzapf and Brown, 2006). Our work supports the notion that inside out activation is not crucial to embryonic fly development, and identifies the major regulatory role of the talin head is in modulating integrin clustering, rather than integrin activation. Furthermore, we demonstrate the biological importance of this mechanism by finding a key role for talin clustering in the reinforcement of Cell-ECM adhesions during a crucial transition in Drosophila development.

Modulation of integrin avidity is a key component of integrin regulation across multiple cellular contexts and cell types. Specifically, in leukocytes, it has been shown that the formation of integrin microclusters precedes ligand binding and that multiple types of avidity interactions are coordinated in these cells to regulate integrin function (Cambi et al., 2006). Furthermore, analyses of loss of function kindlin mutants, another important IAC component thought to function similar to talin in many ways, in platelets found that kindlins play a key role in affecting receptor avidity, but are dispensable for integrin activation arguing that distinct roles of avidity and affinity do exist (Moser et al., 2008). These results, together with the findings presented here suggest that in some specific contexts, integrin avidity regulation can be the main mode of regulating integrin function.

The work we present here fits very well with the conclusions of two cell-culture based studies from Wehrle-Haller and co-workers that explore the role of talin in regulating integrin avidity (Cluzel et al., 2005; Saltel et al., 2009). Specifically, Cluzel et al. demonstrated that the ability of integrins to form clusters was dependent on activated integrins, the talin head domain and interactions with lipids in the plasma membrane.
Importantly, similar to our findings, they also identified integrin clustering as a key upstream factor for the reinforcement of adhesions, as measured by the binding IAC components (Cluzel et al., 2005). Similarly, Saltel and colleagues used mutational analysis and structural modelling to identify the key interacting domains within talin for its function in promoting integrin clustering. They found that a series of residues, or a bridge, spanning the F2-F3 lobes of the talin head is required for the initial recruitment of talin to the membrane in the correct orientation, such that talin’s IBSs can be exposed. Furthermore, it is interactions between this F2-F3 bridge of talin and the plasma membrane that reduce the rotation between the F2 and F3 lobes of the talin head to stably support integrin binding via its F3 domain and therefore integrin clustering and adhesion reinforcement. Our results support similar findings, which suggest that coordination between the F2-F3 lobes of the talin head is essential for integrin clustering. Importantly, we identify a specific residue contributing to this coordination. Finally, the consistency between our findings and those of Saltel and Cluzel despite very different contexts suggests that regulation of integrin avidity, through interactions with the talin head, may be broadly conserved.

It remains unclear exactly what role the G340E mutation, and its effects on the coordination of the F2-F3 lobes, specifically has on the function of talin itself during clustering. We can consider two possibilities. First, perhaps the G340 residue modulates the coordination between the F2-F3 lobes themselves, and the G>E mutation disrupts the necessary conformational changes between the two lobes that allow talin, once bound to the plasma membrane, to induce tilt in integrin cytoplasmic tails. While we cannot completely exclude this possibility, our analysis of an integrin “tilt” mutant (D70R and
G792N) revealed no defects in integrin clustering (Figure 2.5). The second and, in our mind, more likely possibility is that by disrupting the coordination between the F2-F3 domains of the talin head, the G340E mutation affects the ability to talin to be properly recruited to the plasma membrane and thus weakens the interaction between both talin and the plasma membrane, and talin and integrin. This weakened interaction would reduce the ability of talin to act as molecular scaffold, and impinge on the reinforcement of adhesions. However, contrary to what might be expected, we find that in *rhea*<sup>17</sup> embryos talin recruitment to MTJs is not significantly affected. Altogether, our findings imply there may be alternative mechanisms that remain to be uncovered; this represents a fascinating avenue for future study.

Interestingly, in our *rhea*<sup>17</sup> mutant, the inability to form integrin clusters resulted in the inability to reinforce and generate mature integrin adhesions. This, along with the studies previously mentioned, supports the idea that the ability to form robust mature adhesions relies on integrin clustering. Our results support this, as we find in our *rhea*<sup>17</sup> mutant embryos that as development progresses adhesion reinforcement appears to fail, resulting in the severe phenotypes that we observe (Figure 2.2). Specifically, between stages 16-17 of embryonic development, significant growth occurs, and muscle contractions begin. We find that in wild-type embryos to provide resistance to this growing tensile force, IAC components are increasingly recruited to MTJs. However, in *rhea*<sup>17</sup> mutant embryos this reinforcement fails to occur (Figure 2.5), suggesting that perhaps the talin head facilitates this adhesion reinforcement. Furthermore, if adhesion reinforcement does not occur, it has dire consequences for muscle development, as
muscle fail to consolidate into tight compact structures, resulting in longer MTJ’s (Figure 2.5R) and finally, muscle detachment (Figure 2.2D).

In summary, our results provide insights into how integrins are regulated under physiological conditions to give rise to stable tissue architecture. Our work suggests that the canonical model of talin head function as an integrin activator should be modified to include an additional essential role as an orchestrator of integrin clustering and adhesion complex reinforcement. We furthermore illustrate how specific inter-sub-domain interactions in the talin head contribute to the regulation of integrin function. Based on our data we propose the following model for talin function in the fly embryo: talin is recruited to integrin initially through its IBS2 domain (see Ellis et al., 2011), which helps assemble an adhesion complex that links to the cytoskeleton. During embryogenesis, there is an increasing need to generate stronger adhesion as the growth of the embryo generates proportionally greater mechanical strain upon the tissues. It is at this point that clustering becomes essential. Talin is then recruited to integrins via its head domain and is stabilized within growing adhesive contacts by coordinated interactions between F2-F3 and the plasma membrane. These stabilized talin-head-integrin complexes form clusters and act as a scaffold for adhesion complex assembly and cytoskeletal attachment that is maintained and reinforced throughout tissue growth and development. Failure to cluster integrins results in severe defects in reinforcement of cell adhesion, and subsequently Cell-ECM adhesions breakdown in the face of increasing mechanical force. Thus, our work sheds light on the molecular mechanisms that act through talin to promote adhesion receptor clustering and adhesion complex stability, crucial aspects underlying tissue morphogenesis and homeostasis.
2.4 Chapter Two Materials and Methods

2.4.1 Molecular biology

The generation of talinGFP has been previously described (Yuan et al., 2010). To make pUbi-talinEGFP*L334R mutant construct, pBS-talinGFP was mutated using the QuikChange Lightning mutagenesis kit (Stratagene). The talinGFP*L334R cassette was sub-cloned into the pUbi63E vector using a strategy similar to that used to generate the WT talinGFP construct (Franco-Cea et al., 2010). To make pUbi-talinEGFP*K17E mutant construct the same strategy was used. The making of pUASp-GFP-TalinHead was described previously (Tanentzapf and Brown, 2006). This construct was directly mutated to contain the L334R point mutation using the QuikChange mutagenesis kit (Stratagene).

2.4.2 Fly stocks and genetics

All rescue experiments were performed in mutant background such that both maternal and zygotic contributions of talin were eliminated, using the rhea^{79} allele and the Dominant Female Sterile technique (Chou and Perrimon, 1996). The rhea^{79} allele was generated by a P-element excision that covers the entire rhea locus. See (Brown et al., 2002) for a complete characterization. Females of the genotype yw, hs-Flp/++; pUbi-talinGFP, talinGFP*L334R, talinGFP*K17E or headless-TalinGFP/++; rhea^{79a}, FRT2A/OvoD1, FRT2A were subjected to a heatshock-regime during the larval stages to generate a mosaic germline in order to give rise to rhea mutant oocytes with maternally supplied rescued transgenes. Virgins were then crossed to rhea^{79a}/TM6b, dfd-GMR-
nvYFP or rhea\textsuperscript{79a}/TM3, sb, dfd-GMR-nvYFP males. Embryos without the fluorescent balancer were selected for analyses. Using this approach we find that WT talinGFP rescued embryos resemble WT embryos and that over-expression of transgenic talin does not cause any deleterious effects or ectopic signalling of integrin (Ellis et al., 2011; Tanentzapf and Brown, 2006).

The rhea\textsuperscript{17} allele was sequenced according to conventional protocols by sequencing of the entire rhea coding sequence. The G340E mutation was identified in exon 5 through comparison of genomic DNA from homozygous wild type OR flies and heterozygous rhea\textsuperscript{17} flies. Maternal-zygotic rhea\textsuperscript{17} mutants were generated via the Dominant Female Sterile germline-clone technique and crossed to rhea\textsuperscript{17}/TM3, dfd-GMR-nvYFP males.

For all talin FRAP experiments, talinGFP constructs were heterozygous and expressed in a w\textsuperscript{1118} background. For integrin-YFP FRAP experiments, the transgene was either expressed in a heterozygous w\textsuperscript{1118} background (neomycin experiments), or expressed in a rhea mutant background (either rhea\textsuperscript{17} or rhea\textsuperscript{79}).

For analysis of integrin clustering with talin transgenes, yw, hsFLP;;GFP-FRT2A virgins were crossed to males of the genotype of either rhea79, FRT2A/TM3, dfd-GMR-nvYFP, headlessTalin-GFP/Y;;rhea79/TM3, dfd-GMR-nvYFP, L334R; rhea79 FRT2A / TM3, dfd-GMR-nvYFP or rhea17, FRT2A/TM3, dfd-GMR-nvYFP. For analysis of integrin clustering with integrin transgenes ubi-GFP,FRT101; hsFLP males were crossed to
virgin progeny from mys\textsuperscript{XG43}, FRT101/ FM7, Kr>GFP crossed to males of the genotype Ubi-integrin\textit{YFP}*D807R or UBi-integrin\textit{YFP}*G792N. For both integrin and talin transgenes larval progeny were subject to a heat-shock regime in order to induce clones. Wandering third instar larvae were selected for dissection and analysis.

2.4.4 Confocal immunofluorescence imaging and image analysis

Embryos and third instar imaginal wing discs were fixed and stained according to standard protocols. The following antibodies were used in our analysis: rabbit anti-talin (1:500), mouse anti talin (1:50; DSHB) mouse monoclonal anti-βPS-integrin (1:50; DSHB), rat anti-aPS2-integrin (1:200, 7A10), mouse anti-tiggrin (1:1000; Liselotte Fessler, UCLA), mouse anti-Myosin Heavy Chain (1:200; Dan Kiehart, Duke University), rabbit anti-PINCH (1:1000; Mary Beckerle, University of Utah), rabbit anti-phospho-FAK (1:200; Invitrogen) and rabbit anti-paxillin (1:1000; (Yagi et al., 2001)). Rhodamine-conjugated phalloidin (Invitrogen) was used to stain actin filaments (1:400). Fluorescently- conjugated Alexa-Fluor-488, Cy3 and Cy5 secondary antibodies were used at 1:400 dilution (Molecular Probes). Images were collected using an Olympus FV1000 inverted confocal microscope and an UplanFL N 40x 1.30 NA oil objective or a UplanSApo 60x 1.35 NA objective. For all micrographs of whole embryos, or of MTJs, z-stacks were assembled from 8-12 1.0-2.0\textmu m confocal sections. Embryos were staged as described in (Ellis et al., 2013). Recruitment of integrin, talin, or talinGFP to MTJs was calculated according to our previously established method (Ellis et al., 2013; Ellis et al., 2011; Franco-Cea et al., 2010; Tanentzapf and Brown, 2006; Tanentzapf et al., 2006). Briefly, the mean fluorescence intensity of the signal of interest was measured at the MTJ
and the cytoplasm and a ratio of MTJ:cytoplasmic signal was determined. This value was averaged based on measurements of 5 MTJs (all ventral-lateral attachments from hemi-segments A2-A6) from at least 3 embryos. Localization of IAC components to MTJs was quantified as follows, adapted from the method described in (Devenport et al., 2007): for each IAC component (talin, PINCH, and pFAK), recruitment was determined by measuring the mean fluorescence intensity at MTJs, which was then expressed as a ratio over the mean fluorescence of αPS2-integrin staining at each MTJ. For each genotype, at 5 MTJs were measured from at least 3 embryos. MTJ length was assessed by measuring the length:width ratio of the D-V length of ventral-lateral MTJ over the A-P width of adjacent VL1 muscles in the hemisegment posterior to each MTJ that was measured. All images were taken using the same gain and offset settings. This value was averaged based on measurements of 5 MTJs (hemi-segments A2-A6) from at least 3 embryos. All quantitative analyses were obtained using ImageJ (NIH, Bethesda, MD) and two-sided Student’s t-tests were performed using Prism5 (GraphPad Software Inc., La Jolla, CA).

For experiments assaying delay in DC (Figure 2H) timed experiments were performed to obtain embryos ranging from stages 12-17 for phenotypic quantification of dorsal holes as described in (Ellis et al., 2013). Embryos were stained with rhodamine-conjugated phalloidine to visualize morphology for staging.
2.4.5 FRAP

Stage 17 embryos were collected and prepared for FRAP as described previously (Yuan et al., 2010). Briefly, embryos were collected from apple juice plates, dechorinated in 50% bleach for 4 minutes, washed with PBS and mounted onto glass slides in PBS. FRAP analysis was performed at room temperature. Photo-bleaching was performed using a 473 nm laser at 5% power with the Tornado scanning tool (Olympus) for 2 seconds at 100 mseconds per pixel. Fluorescence recovery was recorded over 5 minutes at 1 frame every 4 seconds. To control muscle twitching in and out of focus, multiple regions of interest (ROIs) were selected in non-photobleached regions; only samples for which intensities within control ROIs remained steady throughout the FRAP experiment were used. The mobile fraction and statistical tests were performed using Prism 5 software. Neomycin treatment, which was used to inhibit integrin clustering through sequestration of PI(4,5)P2 phospholids (Arbuzova et al., 2000; Cluzel et al., 2005; Laux et al., 2000), was carried out according to the embryonic drug delivery protocol described in (Schulman et al., 2013), in w^{1118} embryos expressing ubi-integrinYFP (Yuan et al., 2010). The control for integrin FRAP in rhea^{17} zygotic mutant embryos was rhea^{79/+} embryos expressing ubi-integrinYFP. We have previously observed that genetic background influences the baseline turnover of integrin adhesion complex components and therefore each experiment requires its own control (Pines et al., 2012). In particular, we find that the drug delivery protocol lowers turnover in vehicle-only treated embryos, and thus explains the differences in the mobile fractions of the controls shown in Figure 2.2g versus Fig. 2.2h.
2.5 Chapter 2 Figures

Fig. 2.1 Integrin-binding to the talin head, but not integrin activation, is essential for talin-mediated integrin function in *Drosophila* development. (a-d) Confocal z stacks of maternal zygotic talin null embryos rescued with either full-length WT talinGFP transgene (a), talinGFP*L334R mutant transgene (b), talinGFP*K17E (c) or headless talinGFP transgene (d) and stained for F-actin (green) and bPS-integrin (magenta). Scale
Recruitment of ubi-promoter driven full-length WT talinGFP and talinGFP*L334R to sites of adhesion was assayed in talin null (e) and in wild-type embryos (f). Compared to WT TalinGFP, TalinGFP*L334R was well recruited in a background devoid of any endogenous talin (e; **p<0.01), but competed less well in the presence of endogenous talin and was only weakly recruited to sites of adhesion compared to WT, which was robustly recruited (f; ***p<0.001). (g) FRAP experiments on WT talinGFP and talinGFP*L334R reveal that talinGFP*L334R is much less stable at sites of adhesion than WT talinGFP. (h-i) Recruitment of ubi-promoter driven full-length WT talinGFP and talinGFP*K17E to sites of adhesion was assayed in talin null (h) and in wild-type embryos (i). Compared to WT TalinGFP, TalinGFP*K17E had no significant changes in recruitment to MTJ’s regardless of background. (j) FRAP experiments on WT talinGFP and talinGFP*K17E reveal that talinGFP*K17E is much less stable than talinGFP at sites of adhesion. (k-l) Recruitment of ubi-promoter driven, GFP-tagged full-length WT talin and headless talin to sites of adhesion was assayed in a talin null background (k) and in wild-type embryos (l). In a WT background, headless-talinGFP competed less well with endogenous talin and was only weakly recruited to sites of adhesion compared to WT (***p<0.001); in the absence of endogenous talin, headless-talin was well recruited to sites of adhesion. (m) FRAP experiments on talinGFP-WT and headless-talinGFP reveal that headless talin is much less stable at sites of adhesion than talinGFP-WT.
Fig. 2.2 rheal encodes a hypomorphic allele of talin which disrupts talin head function. (a-b) The rheal allele is characterized by a missense mutation in a conserved glycine residue in the F3 lobe of the talin head FERM domain, G340E. (a) Alignment of residues 320-360 of fly talin F3 domain with human talin isoforms. Dark blue indicates identical residues between homologues, lighter blue indicates similar residues. The mutations identified in rheal are indicated with an arrowhead. (b) Schematic of key
domains in talin for this study. The talin head is contains an N-terminal atypical FERM domain (Elliott et al., 2010) and a C-terminal rod domain comprised of 13 helical bundles (Goult et al., 2013). (c-d) Whole mount stage 17 embryos stained for F-actin (green) and integrin (magenta) reveal that rhea17 mutant embryos (d) harbour severe morphogenetic phenotypes in GBR (e) and DC (f), as well as muscle detachment defects (g) compared to WT heterozygous embryos (c,e-g). (h) Embryos with the rhea17 mutation, heterozygous for rhea17 and talin null embryos were scored for dorsal holes at stage 13-17. rhea17 appears to have a milder delay in DC than talin null. (i) αPS2-integrin recruitment was measured in WT (i,i’) and rhea17 (i,i**) stage 16 embryonic muscles stained for integrin. Integrin was recruited at WT levels in rhea17 embryos. (j) Talin recruitment was measured in WT (j,j’) and rhea17 (j,j**) stage 16 embryonic muscles stained for talin. Talin was well recruited in rhea17 embryos. Scale bars: c-d =100µm; i-j; j-k = 20µm.
Fig. 2.3 *rhea*<sup>17</sup> disrupts integrin clustering. (a-b) Recruitment of integrins to MTJs was measured in stage 16 and stage 17 for both control (a) and *rhea*<sup>17</sup> mutant embryos (b). In contrast to control embryos (**p<0.001), *rhea*<sup>17</sup> mutant embryos did not exhibit an increase in integrin recruitment to MTJs during this developmental transition. (c-c') Clones of cells lacking talin, marked by the absence of GFP (c), failed to cluster integrins into adhesions (c'). (d,d') Clones of cells expressing the *rhea*<sup>17</sup> mutant allele of talin (marked by absence of GFP in (d) also failed to cluster integrins (d'). (e-f) Expression of a full length talin point mutant that specifically disrupts IBS-1 binding (e, talinGFP*R367A, LI>AA, see Ellis et al, 2011) or that specifically disrupts integrin activation (f, talinGFP*L334R) restored integrin adhesions (e', f') within the clones of cells (arrow) lacking endogenous talin and the GFP marker (e,f). The red outline demarcates the position of the clones. (g-h) FRAP analysis revealed the mobile fraction of integrin-YFP was higher than respective controls in embryos treated with neomycin (h; **p<0.001) or in *rhea*<sup>17</sup> zygotic mutant embryos (g; **p<0.001). Since these two FRAP experiments employed different genetic backgrounds and protocols in preparation for FRAP (ie. embryos in h were subjected to a drug delivery protocol), they necessitated two separate controls. In h, the control was established from vehicle-treated wild type embryos expressing integrin-YFP. In g, the controls was taken from heterozygous talin mutant embryos.
Fig. 2.4 *rhea*<sup>17</sup> disrupts adhesion complex reinforcement and adhesion consolidation. WT and *rhea*<sup>17</sup> embryonic muscles stained for talin (red in a-d; grey in a’-d’) and integrin (green in a-d) at stage 16 (a-b) and stage 17 (c-d). (e) The recruitment of talin to adhesions (normalized to integrin levels; see materials and methods) was comparable
between WT and rhea$^{17}$ in stage 16 embryos. However, although talin was maintained at sites of adhesion, its recruitment was not reinforced in rhea$^{17}$ embryos in stage 17 embryos (e). (f-j). WT and rhea$^{17}$ embryos stained for integrin (green in f-i) and PINCH (red in f-i, grey in f$^{-}$-i$'$) at stage 16 (f-g) and stage 17 (h-i). PINCH recruitment was not reinforced in stage 17 rhea$^{17}$ embryos as determined by measuring the ratio of α-PINCH fluorescence intensity relative to integrin intensity at MTJs. (j; see Materials and Methods). (k-o) WT and rhea$^{17}$ embryos stained for integrin (green in k-n) and pFAK (red in k-n; grey in k$'$-n$'$). pFAK recruitment was not reinforced in stage 17 rhea$^{17}$ embryos as determined by measuring the ratio of α-pFAK fluorescence intensity relative to integrin intensity at MTJs (o; see Materials and Methods). (p-r) MTJ length was measured in control heterozygous (p) and rhea$^{17}$ mutant (q) embryos (see materials and methods). MTJs were significantly longer in rhea$^{17}$ mutants compared to control embryos (****p<0.0001). Scale bars: a-n =50µm; p-q = 10µm.
Fig. 2.5 G340 maintains an intermolecular interaction between F2 and F3 that couples their activity. (a) The conserved role of G340 (G331 in mammalian talin2, shown here) is to stabilize the domain orientation of F2 and F3 (a), which work together to induce integrin activation and stabilize that talin head at the plasma membrane. Modelling based on known structures (see (Anthis et al., 2009)) of mouse talin2 and the integrin cytoplasmic tail suggests that the G340E mutation would disrupt the tight apposition of F2 and F3, thus allowing them to behave as independent modules. (b-e) Mosaic analysis of integrin mutant in which βPS–integrinYFP transgenes containing either the D807R (b) or G792N (c) point mutations were ubiquitously expressed. Both mutations rescued the formation of basally localized integrin clusters (b', c') in integrin mutant clones (marked by loss of GFP in b,c ). (d,e) Quantification of the density of basal integrin clusters failed to reveal any significant differences between the density of clusters in control tissue versus mutant tissue expressing either β-integrinYFP*D807R (h'') or β-integrinYFP*G792N (h''). Scale bars: a-b = 20µm. c-h =10µm (f-g) The recruitment of talin was measured in neomycin treated embryos (f) and in rhea17 embryos (g). Talin recruitment was significantly reduced in neomycin-treated compared to controls (*p<0.05). In contrast there was no such reduction in the rhea17 embryos suggesting the G340E mutant talin protein interacts with the membrane as well as the WT protein.
CHAPTER THREE: Investigating the Role of Integrin-Mediated Adhesion in Apical Cell Oscillations During DC

3.1 Chapter Three Introduction

Morphogenesis is one of the key programs elemental to the development and homeostasis of complex organs and tissues. Additionally, morphogenesis is required for the establishment of the three dimensional structures in multicellular development. During morphogenesis a series of stereotypical structural rearrangements occur that are driven by a standard set of cell behaviours coordinated across tissues (Walck-Shannon and Hardin, 2014). These cell behaviours require the ability to generate and resist biomechanical force within cells. Many different molecular mechanisms are known to generate biomechanical force within cells during morphogenesis. Current studies of morphogenesis aim to provide a comprehensive view into how cell-level molecular mechanisms can be coordinated across tissues to drive the large-scale tissue movements and rearrangements that characterise this process.

A well-studied cell behaviour that contributes to morphogenesis is the reduction of the apical surface of the cell, or apical cell constrictions (Martin and Goldstein, 2014; Martin et al., 2009). The steady reduction of the apical surface of a cell can have a variety of consequences for tissue geometry and therefore can contribute to many changes including tissue bending, folding and ingression (Sawyer et al., 2010). Apical constrictions therefore underlie many developmental processes across phyla, including
xenopus gastrulation, vertebrate neural tube closure and *Drosophila* DC. Although the geometric changes characterizing apical constrictions are the same, the mechanisms that cause this change may not be. A common mechanism driving apical cell constrictions is the localization an active actomyosin network to the apical surface of cells. Furthermore, work in *Drosophila* has demonstrated that this activity can be spatially coordinated across cells, and that pulses of myosin activity can drive apical constrictions in a ratchet-like manner to achieve robust morphogenesis (Martin et al., 2009).

In order for the cell behaviours driving morphogenesis to result in tissue wide changes and movements, cells must be connected to each other and their external environment. Cell adhesion is therefore a crucial module of morphogenesis. The main family of proteins responsible for connections between cell and their underlying extracellular matrix (ECM) are integrins. Integrins are transmembrane type 1 adhesion receptors that provide a link between the ECM and the actin cytoskeleton. In order to achieve their roles in morphogenesis, integrin receptors mediate many signalling cascades within the cell. They achieve this via their intracellular domains where they bind a diverse group of proteins known as the IAC that is responsible for mediating downstream signals and connect directly to actin. Integrins are heterodimers composed of one α and one β subunit. In flies, 10 possible heterodimers can be formed with 5α and 2β subunits, in contrast to mammals, which can form up 24 possible heterodimers with 18α and 8β subunits. However there is strong sequence homology, making flies a simpler but still meaningful system in which to study integrin function.
During fly embryogenesis, Dorsal Closure (DC) is an integrin dependent event that requires spatially and temporally coordinated tissue movements and serves as a model system in developmental biology to study the role of biomechanical force during morphogenesis. During DC, two encroaching epithelial cover over an extra-embryonic epithelium known as the AS to seal an eye-shaped wound in the dorsal side of the embryo. The process of DC is extremely robust and is driven by many redundant cellular behaviours including: contractile force in the AS due to cortical actomyosin networks in AS cells, a supracellular actomyosin purse string, resistive forces by the lateral epidermis, adhesive zipping and coordinated apoptosis in the AS, increasing the tension within the tissue (Gorfinkiel et al., 2009; Rodriguez-Diaz et al., 2008; Solon et al., 2009; Toyama et al., 2008). Recent studies have suggested that AS alone can drive closure, and that tension within the AS is generated via multiple redundant mechanisms (Wells et al., 2014).

Importantly, loss of integrin adhesion results in severe phenotypes during DC, including failure. This manifests in tearing between the AS and the underlying yolk, highlighting the importance of adhesion between the AS and its underlying ECM (Narasimha and Brown, 2004). Loss of integrin function is further associated with changes in stereotypic cellular behaviour known to contribute to the completion of DC which manifests in phenotypes including; altered geometry of the dorsal opening, reduced seam formation, and alterations in patterns of apical oscillations within the AS (Gorfinkiel et al., 2009; Peralta et al., 2007).
Importantly, previous work from our lab identified novel adhesive structures on the basal surface of the AS and described a previously uncharacterized role for cell-ECM adhesion in coordinating the transmission of force across the AS during DC (Goodwin et al, personal communication). Perturbation of integrin function leads to changes in cell to cell force transmission across the apical plane of the tissue. It was hypothesized that the apical pole of AS cells is tethered to the substrate via apical-basal mechanical coupling and cell-ECM adhesions; thus, apically-generated forces which drive cell oscillations are subject to passive resistance from basal adhesions to the ECM. However, links between integrin-mediated adhesion and actomyosin machinery that drives apical constrictions were not explored.

Here we show that integrins are required for proper apical oscillations by maintaining the activity of actomyosin machinery during DC. Decreased integrin adhesion resulted in decreased myosin flow frequency and speed. These changes are correlated with changes in the localization and stability of junctional protein cadherin, which anchors the actomyosin machinery to the membrane. Overall, these results demonstrate a novel way for integrin-mediated Cell-ECM adhesion to regulate actomyosin based force generating mechanisms during morphogenesis.
3.2 Chapter Three Results

3.2.1 Loss of basally localized integrins in the amnioserosa affects the amplitude of apical cell oscillations

Integrins in the AS are known to be localized to the lateral junctions as well as basally in AS (Narasimha and Brown, 2004). In order to confirm the localization of integrins during DC, we imaged cross-sections of wild-type (Figure 3.1A) and integrin null (Figure 3.1B) embryos. βPS-integrin strongly localized to the basal surface of the AS; weaker integrin signal was also detected in the lateral domain. (Figure 3.1 A, A’’). This localization pattern was lost upon genetic removal of βPS using the dominant-female sterile germline clone technique (Chou and Perrimon, 1996)(Figure 3.1 B, B’’). This suggests that integrin is primarily localized to the basal surface of the AS.

Previous work has implicated integrin adhesion in cell behaviours associated with DC, including perturbations upon the ability of AS cells to apically oscillate, a crucial force driving DC (Gorfinkiel et al., 2009; Narasimha and Brown, 2004), Goodwin et al, personal communication). We therefore sought to quantitatively analyze the effect of integrin loss on apical cell oscillations during DC. In order to measure cell oscillations, we utilized customized MatLab scripts to trace cell perimeters, as marked by tagged E-cadherin-GFP (cadGFP) in the AS. We could therefore quantitatively visualize and measure the movement of AS cell perimeters during DC (Figure 3.1C). We found that the period of cell oscillations is unchanged with loss of integrin (myospheroid (mys)) (Figure 3.1D), as compared to both wild-type and heterozygous controls. Interestingly, however, the amplitude of oscillations is significantly increased when integrin adhesion is lost (Figure 3.1E). This dynamic change in oscillation behaviour is also reflected in the
overall distribution of cell size of in the AS when measured in fixed tissues, which we find to have smaller area and radius (Figure 3.1F, G). Interestingly, we find a biphasic change in cell radius between integrin heterozygous versus wild type and null animals suggesting perhaps levels of integrin need to be precisely regulated for correct cell shape. We therefore find that loss of integrin, a predominantly basally-localized adhesion protein, affects apical cell oscillations in the AS specifically by perturbing their amplitude of oscillations.

3.2.2 Myosin speed is reduced in integrin mutants

Apical constrictions have been well characterized in vivo. First, the actomyosin network, visualized as a myosin pulse, contracts the apical surface of the cell, which significantly reduces the cell area. This is followed by a period of stabilization, and it is through these phases of rapid constriction and stabilization that apical constrictions occur (Blanchard et al., 2010; David et al., 2010; Martin and Goldstein, 2014; Martin et al., 2009) (Figure 3.2C). Apical cell constrictions have been shown to occur in a pulsatile manner during DC (Gorfinkiel et al., 2009) and DC is known to be dependent on MyoII activity (Franke et al., 2005). We therefore visualized actomyosin dynamics in the AS using a functional fusion between Drosophila MRLC (spaghetti-squash, sqh) and mCherry, called sqh-mCherry, and CadGFP to visualize cell outlines. We performed analyses in wild-type (w1118), and integrin heterozygous and integrin null animals (Figure 3.2A and B, respectively). We found no significant differences in any of the behaviours measured between wild-type and integrin heterozygous backgrounds, and therefore utilized heterozygous embryos as controls for the remainder of our analysis. We
utilized particle image velocimetry (PIV) based on an approach previously described (Levayer and Lecuit, 2013) to generate vector fields of myosin flows, and cell outlines to isolate PIV vectors corresponding to individual cells (Figure 3.2 D, see materials and methods). These vectors of myosin flow can then be used to calculate standard measurements of myosin flows. Myosin flows describe medial myosin movement, including myosin coalescence and divergence such as has been characterized to drive apical cell oscillations.

When we analyzed myosin flows we found that loss of integrin had a very specific effect. We found that the mean instantaneous speed, or the mean magnitude of all the vectors regardless of direction (see materials and methods) of myosin flows was significantly decreased in integrin mutants. Importantly, previous analyses have found a correlation between decreased apical area and decreased myosin speed, associated with a mechanical balance of force across the cell during germband elongation (Levayer and Lecuit, 2013). However, unlike the genetic backgrounds analyzed in the previous study, we did not find that myosin intensity was significantly different in our mutant background (Figure 3.2G), although this difference can perhaps be attributed to the differences between germband elongation (GBE), which occurs in one direction, and is driven by cell intercalation versus DC which involves tissue contraction to a single point and isotropic deformations associated with apical constrictions.

3.2.3 Myosin polarization is unaffected by integrin loss

Given that medial apical myosin is known to flow towards junctions during DC (Blanchard et al., 2010; David et al., 2010) and that previous analysis had suggested that
decrease in myosin speed correlated with symmetric pulling forces across cells (Levayer and Lecuit, 2013), we chose to analyze the polarity of myosin flows during DC using PIV. Interestingly, we found that myosin flows were not polarized during DC (Figure 3.2F) agreeing with the isotropic apical cell deformations and tissue contraction associated with DC (Gorfinkiel et al., 2009). We quantified this by comparing the absolute instantaneous speed of vectors in the x (Figure 3.2H, |u|) versus the absolute instantaneous speed in the y (Figure 3.2I, |v|) direction. We can express this as a ratio (|u|/|v|), in which a value other than one demonstrates polarization. However, we found that all our experimental conditions were not significantly different from one (Figure 3.1J), suggesting that myosin flows are not polarized, and that loss of integrin does not affect this lack of polarization.

3.2.4 The subcellular localization of cadherin-based junctions is disrupted in integrin mutants

E-cadherin polarization has been shown to mediate medial actomyosin pulling forces during axis elongation in Drosophila (Levayer and Lecuit, 2013). Furthermore, integrin adhesion complexes are known to interact with cadherin complexes during morphogenesis (McMillen and Holley, 2015). We hypothesized that change we observed in myosin speed due to integrin loss may be due to defects in cadherin localization in cells in the AS. We found that loss of integrin resulted in aberrant e-cadherin localization (Figure 3.3A compared to Figure 3.3B). Specifically, we observed that in wildtype AS cells E-cadherin localized to discrete puncta along the cell perimeters, whereas in integrin mutants the puncta appeared brighter, and less frequently. We quantified this observation
by measuring cadherin intensity along cell outlines (Figure 3.3C,D). We used these measurements of intensity to define puncta as peaks of intensity that were at least one standard deviation above the average intensity of the cell outlines (peaks marked by pink circles in Figure 3.3 C, D). Using this analysis we found that in integrin mutants puncta were brighter on average (Figure 3.3E) and also that the maximum intensity of peaks was higher in integrin mutants (Figure 3.3F). However, despite the increased brightness of the peaks in integrin mutants, we found that they occurred significantly less often (Figure 3.3G). This quantification therefore supported our observations that subcellular localization of cadherin-based junctions is perturbed in integrin mutants.

3.2.5 The kinetics of cadherin turnover are altered in integrin mutants

We sought to measure if the observed effects on cadherin localization would alter the stability of E-cadherin at the cell membrane. In order to measure cadherin turnover we utilized FRAP of embryos expressing CadGFP (Figure 3.3H). We found that the half time ($t_{1/2}$) of recovery of E-cadherin at the cell membrane in integrin mutants was significantly reduced compared to controls (Figure 3.3I). Surprisingly however, we found that the overall turnover, as measured by the mobile fraction, was the same between integrin mutants and controls (Figure 3.3J). This suggests that although the mobility of cadherin is unaffected by integrin loss, the turnover of cadherin is occurring at a faster rate. This difference in kinetics may underlie the changes in cadherin localization we see in integrin mutants.
3.2.6 Structure function mutations in integrin reveal a phenotypic variety of DC defects

In order to gain mechanistic insight into our observations for integrin in cell behaviours during DC we utilized integrin point mutants perturbing various aspects of integrin function previously characterized by our lab (schematic in Figure 3.4D)(Ellis et al., 2011; Pines et al., 2011). We hypothesized that different modes of integrin regulation may be required for DC, and that specific domains in integrin may mediate its contribution to cadherin localization and apical cell oscillations during development. Importantly, we initially verified that we were able to recapitulate the penetrance of phenotypes in three different integrin dependent events during Drosophila embryogenesis (Figure 3.4A-C). The first two, GBR and DC rely on dynamic integrin adhesions (Figure 3.4A and B, respectively), while the second, muscle attachments, relies on long-term stable adhesions (Figure 3.4C). We found slight differences in penetrance of phenotypes as compared to previous analyses (Ellis et al., 2011; Pines et al., 2011). We hypothesize that this may be a result of the qualitative nature of these measurements, and importantly, our findings for integrin null and wild-type were consistent with previous observations (Figure 3.4A-C, (Ellis et al., 2011; Pines et al., 2011)).

In order to more closely examine the role of different modes of integrin regulation during DC, we utilized an assay previously designed in our lab to measure delays during DC (Ellis et al., 2013). Specifically, we assayed for the presence of open dorsal holes during specific stages of embryonic development, as revealed by stereotypic developmental events such as gut morphogenesis and muscle formation. In wild-type embryos, DC is completed by stage 15, and only 5% of wild-type embryos have open
dorsal holes, as compared to 77% in integrin mutants (Figure 3.4F). We can therefore use this assay to investigate more penetrant DC defects.

We first investigated the role of the extracellular domain of integrin for DC. We utilized a point mutant integrinYFP*S196F, which is a deactivating mutation that renders integrin unable to undergo conformational changes associated with ligand binding and therefore perturbs outside-in integrin signalling (Chen et al., 2006a; Chen et al., 2006b; Hogg et al., 1999; Pines et al., 2011). We found a severe DC defect, and a delay in DC that was stronger than that observed for total integrin loss suggesting perhaps a dominant negative effect (Figure 3.4G).

We hypothesized that given the requirement for correct intracellular cadherin localization that the intracellular cytoplasmic tail of integrin, which contains many protein-protein binding regions, would be key to DC, and perhaps regulation of apical oscillations. We therefore investigated multiple mutations in this region of βPS-integrin. Importantly, a complete truncation of the βPS cytoplasmic tail, integrinYFP*804stop results in a severe DC defect (Figure 3.4B), and delay in DC (Figure 3.4H), suggesting that the cytoplasmic tail is crucial to DC. However, when we looked at specific point mutants within the tail itself, we found that the phenotypes were generally less severe than the complete loss of the tail domain. Specifically, we observed that integrinYFP*N828A, which is a mutation in the proximal NPxY motif of integrin that is hypothesized to reduce talin binding via the talin IBS-1 domain (Calderwood et al., 2002; Ellis et al., 2011; Wegener et al., 2007), did have a severe phenotype (Figure 3.4B), but with less of delay than complete integrin loss (Figure 3.4J). By contrast, an activating mutation, integrinYFP*Y831F/Y843F, designed to promote the binding of talin (Anthis
et al., 2009) almost completely rescued integrin loss (Figure 3.4B) and had a very minimal delay in DC (Figure 3.4K). We found that only one mutation, integrinYFP*N840A, resulted in a similar severity of both delay and penetrant phenotypes (Figure 3.4B, J). This mutation is a deactivating mutation that is hypothesized to perturb the ability of the kindlin protein to bind to integrin cytoplasmic tails (Harburger et al., 2009; Moser et al., 2008; Pines et al., 2011). We therefore chose to investigate this mutation in further detail.

3.2.7 IntegrinYFP*N840A does not affect apical cell oscillations and is not required for cell movement during DC

Previous work from our lab identified a role for cell movement and force transduction during DC (Goodwin et al, personal communication). Specifically, during oscillations in early stages of DC the geometric centroids of AS cells moved in a random motion manner, and that this movement was increased with integrin loss. In order to exclude the possibility that the severe delay and phenotype associated with integrinYFP*N840A was due to this previously described trend, we measured cell movement in integrinYFP*N840A mutants (Figure 3.5A-E). We did not observe any significantly different trends in cell movement with integrinYFP*N840A as compared to controls (Figure 3.5 A-E). We therefore measured the effect of integrinYFP*N840A on cell oscillations. Surprisingly, we found that integrinYFP*N840A did not perturb amplitude of cell oscillations (Figure 5F) or cell oscillation period (Figure 3.5G), suggesting that DC defects arising from integrinYFP*N840 rescue is independent of all previously identified mechanisms of integrin-mediated adhesion during DC. This
suggests a role for kindlin in regulating dynamic integrin adhesion that is outside the scope of this study, but would be an interesting area of future research. Consistent with this hypothesis, integrinYFP*840 gives rise to phenotypes in GBR, another dynamic morphogenetic movement.

3.3 Chapter Three Discussion

Here we have identified a role for integrin in modulating apical cell oscillations during DC. Specifically, we find that loss of integrin-mediated adhesion results in an increase in the amplitude of apical cell oscillations, but that the period remains unchanged (Figure 3.1). We find that this change in cell oscillation dynamics correlates with a decrease in speed of myosin flows within oscillating cells (Figure 3.2). Finally, similar to previous analyses (Levayer and Lecuit, 2013), we are able to correlate these changes in myosin flows with perturbed cadherin localization and kinetics of turnover (Figure 3.3). We interrogated a possible mechanism by screening integrin point mutants that perturb specific aspects of integrin function, and found a diversity of DC phenotypes; the strongest phenotype identified did not correlate with either apical cell oscillations or our previously identified role for cell movement (Figure 3.4 and 3.5).

Given their many common roles, such as in morphogenesis and cell migration, it is unsurprising that cadherins and integrins are known to undergo crosstalk both in vitro and in vivo settings (Burute and Thery, 2012; McMillen and Holley, 2015; Weber et al., 2011). However, the precise molecular interactions and/or signalling events allowing for connection between integrin and cadherin remain unclear, and evidence is emerging that these interactions are dependent upon the physical context in which they occur (See Chapter 4.3). This underscores the importance of studying these interactions in vivo,
where precise control over cell adhesiveness in a three dimensional environment is crucial.

This work aims to expand on this prior research by providing another in vivo example of integrin and cadherin cross talk, and demonstrate a role for this in mediating morphogenesis. Interestingly, previous work from our lab (Goodwin et al, personal communication) demonstrated that integrin mediated adhesion in the AS is essential for force transmission during DC. Furthermore, genetically increasing the amount of cell-ECM adhesion resulted in less efficient force transmission across the apical plane of the tissue. Interestingly, eliminating cell-cell adhesion resulted in an upregulation of cell-ECM adhesions, resulting in less efficient cell-cell force transmission. This demonstrated that integrin and cadherin in the AS are linked and contribute to force transmission. Here the first time, however, we demonstrate the mechanism that might be responsible for that linkage, in which cadherin localization, and/or turnover kinetics are being altered by integrin loss, resulting in changes in myosin dynamics that in turn affect the cell behaviours that drive morphogenesis.

It remains unclear what role integrin and cadherin crosstalk have to play in regulating myosin flows during DC. A recent study by Levayer at al demonstrated that localization of cadherin complexes played an important role in setting medial actomyosin pulling forces on junctions (Levayer and Lecuit, 2013). Thus spatial control over the localization of cadherin complexes was crucial to orienting myosin flows, as flows of myosin became planar polarized during GBE, preferentially directing the shrinkage of DV junctions (Levayer and Lecuit, 2013). Importantly, previous work from this same
group has observed that perturbing cadherin endocytosis results in defects in cell intercalation, the morphogenetic cell behaviour driving GBE, and more specifically that the localization of cadherin is controlled by endocytosis, modulated by myosin based signalling (Levayer et al., 2011). Thus, during GBE myosin flows are part of a negative feedback loop, in which cadherin turnover directs and is perpetuated by myosin flows to drive junction remodelling during intercalation, which can act to spatially localize cadherins through endocytosis, such that myosin flows can preferentially pull on specific junctions to drive cell shapes changes underlying cell intercalation. These findings share many similarities to the relationships between cadherin, integrins and myosin we observed during DC. Specifically, in both GBE and DC, correlations can be observed between defective cadherin localization or turnover, and changes in myosin flows. Furthermore, these changes in cadherin localization may be driven by changes in the endocytic machinery, resulting in changes in cadherin kinetics at the membrane.

However, different cellular behaviours drive GBE and DC; in GBE, cell area is relatively constant and junctions are remodelled to allow for intercalation. During DC, cells contract anisotropically. Importantly, we observe that cadherin localization during DC in wildtype embryos is distributed evenly along the cell membrane in discrete puncta, whereas in integrin null embryos, these puncta become brighter and occur less often (Figure 3.3). Perhaps the diffuse localization of cadherin around AS cells allows pulling forces across the cell to be uniform. However, when integrin adhesion is lost, cadherins become mislocalized, and myosin cannot generate sufficient tension via adherence to the cell membrane in order to pull robustly. This results in slower speeds and inefficient
myosin flows, leading to the fluctuations in amplitude of cell oscillations. However, despite the clear consequences of decreased myosin speeds on morphogenesis, it remains unclear why myosin flows are slower. We hypothesize these inefficient myosin flows are a consequence of defects in actin architecture, which is supported by previous studies identifying the importance of actin regulators in myosin pulses (Levayer and Lecuit, 2013). Interestingly, we do seem to observe changes in the architecture of the actin cytoskeleton upon integrin loss (Figure 3.3A,B). Furthermore, we have mutations in talin available that specifically disrupt talin binding to actin (Franco-Cea et al., 2010). It would an interesting area of future research to investigate the role of integrin/cadherins connections to actin in mediating actin architecture in the AS, and to explore any possible consequences for myosin’s ability to mediate pulling forces in cells during DC.

3.4 Chapter Three Materials and Methods

3.4.1 Molecular biology

The construction of all transgenic pUBI-bPS-integrin:YFP transgenes has been described elsewhere (Pines et al., 2011).

3.4.2 Fly stocks and genetics

βPS-integrin null mutants were generated using the Dominant Female Sterile germline clone technique (Chou and Perrimon, 1996) in order to remove both maternal and zygotic contributions of integrin. Virgins of mysXG43, FRT101 were crossed to males of ywhsF1p,OvoD1, FRT101 (Bloomington Stock Center, #1813) and the larval progeny
were subjected to a heatshock regime to generate a *mys* mosaic germline. Virgins were then crossed to *dfd-GMR-nvYFP* males. In the case of rescue experiments virgins were crossed to males of the genotype *dfd-GMR-nvYFP; integrinYFP*(WT or mutants)/+. Mutant embryos were identified by lack of the fluorescent marker, and mutant rescues by the additional presence of integrinYFP. Embryos carrying the fluorescent marker (ie. heterozygous *mys*+/siblings) were used as controls, where marked.

The following markers were used for live imaging: *DE-Cad-mTomato* (gift of N. Gorfinkiel, Consejo Superior de Investigaciones Cientificas-Universidad Autonoma de Madrid, Madrid, Spain), *ubi-DE-cadherin:GFP* (Oda and Tsukita, 2001), *sqh-sqh:mCherry* (Martin et al., 2009).

### 3.4.3 Confocal immunofluorescence imaging

Embryos were dechorionated in 50% bleach for ~4 minutes and staged according to the criteria of (Ellis et al., 2013). For live imaging embryos were then aligned and glued to a 1mm coverslip using embryo glue (Scotch double-sided tape dissolved in heptane), dorsal side down. Coverslips were mounted in halocarbon oil (Sigma) on glass slides with a cover-slip bridge to prevent compression of the embryos. For fixed imaging embryos were fixed in 4% PFA according to standard protocols. For ventral views of embryos (Figure 3.1), the following protocol was modified. Briefly, embryos were fixed and stained as usual, then fixed again, and subjected to a glycerol series. They were then cut and mounted on a cover slip, using a coverslip bridge. The following antibodies were used in our analysis: mouse monoclonal anti-bPS-integrin (1:50; DSHB), rat monoclonal anti-DE-cadherin (1:40; DSHB), Rhodamine-conjugated phalloidin (Invitrogen) (1:400).
Fluorescently-conjugated Alexa-Fluor-488, Cy3 and Cy5 secondary antibodies were used at 1:400 dilution (Molecular Probes).

Images were collected using an Olympus FV1000 inverted confocal microscope and an UplanFL N 40x 1.30 NA oil objective or a UplanSApo 60x 1.35 NA objective, or a Zeiss Axiovert 200M spinning disk confocal microscope using a 63X 1.40 NA or 100X 1.45 NA objective. All images were acquired maintaining consistent exposure time, laser power, gain, and offset settings between control and experiment embryos to allow for direct comparison. For movies of cell oscillations and myosin flows, about 5-10 1 µm confocal sections were collected at 20-second intervals for a 20 minute time period. At least 5 movies were taken of each genotype. For fixed images of cells, z-stacks were assembled from 10 0.5 mm confocal sections. Animations were assembled and processed using ImageJ (NIH, Bethesda, MD), and images were processed to span the full range of the grey-scale using Adobe Photoshop. All quantitative analyses were obtained using two-sided Student’s t-tests were performed using Prism5 (GraphPad Software Inc., La Jolla, CA).

For experiments assaying delay in dorsal closure (Figure 3.5) timed experiments were performed to obtain embryos ranging from stages 12-17 for phenotypic quantification of dorsal holes as described in (Ellis et al., 2013). Embryos were stained with rhodamine-conjugated phalloidin to visualize morphology for staging.
3.4.4 FRAP

Stage 14 embryos were collected and mounted for live imaging as described above. FRAP analysis was performed at room temperature. Photo-bleaching was performed using a 473 nm laser at 10% power with the Tornado scanning tool (Olympus) for 2 seconds at 100 mmseconds per pixel. Fluorescence recovery was recorded over minutes at 1 frame every 4 seconds. To control for drift of embryos, multiple regions of interest (ROIs) were selected in non-photobleached regions; only samples for which intensities within control ROIs remained steady throughout the FRAP experiment were used. The mobile fraction and statistical tests were performed using Prism 5 software.

3.4.5 Image analysis

Image analysis was done using custom tools generated in MatLab. In brief, cell outlines were obtained from movies with labeled cadherin and processed in SIESTA (Fernandez-Gonzalez and Zallen, 2011) to obtain segmented cell outlines. These generated outlines were then processed in MatLab to calculate cell area and centroid over time. Cell area curves were normalized and detrended to obtain measurements of the amplitude and period of cell oscillations.

Particle image velocimetry (PIV) was performed using custom-written MatLab scripts based on the approach described in (Levayer and Lecuit, 2013) in order to measure movement of fluorescently tagged myosin. In brief, images were divided into square interrogation windows that were 16 pixels squared, and overlapping by 75%. The 2D cross correlation of each window for each frame over the duration of the movie was used to calculate the direction and magnitude of intensity movement. Cell outlines were
calculated as described above and used to isolate PIV vectors corresponding to individual cells. PIV vectors were then used to compute mean instantaneous speed along the x (AP) and y (ML) axes and the magnitude of the vectors was used to compute mean instantaneous speed. The mean for a cell was defined as the mean of the speeds described by all vectors within the cell. To compute |u|/|v|, we took the ratio of the absolute value of each vector’s x component (|u|) to the absolute value of the y component (|v|). The angle of vectors was measured with respect to the x (AP) axis, and the distribution of all PIV vector angles was visualized using rose diagrams generated with MatLab.

Measurement of Cadherin Intensity along cell edges was done using custom MatLab scripts. The script used a user-generated cell outline and refined it using a series of morphological image processing steps to ensure accuracy and remove user bias. The resultant outline coordinates were used to determine cadherin intensity at each point along the outline. For each point, pixel brightness values were determined for a square region of 12 by 12 pixels around the point. Pixels with brightness greater than one standard deviation of the mean brightness (computed from the entire image) were included in intensity calculation. The mean of all included pixels was used as the intensity value for that point along the outline. The resultant curve of intensity vs. position along the cell outline was used to compute the magnitude and spacing of intensity peaks. Peaks greater than one standard deviation above the mean intensity of the entire cell outline were included. If two peaks were not separated by a trough in which intensity fell below the mean, they were counted as a single peak, with magnitude equal to that of the taller of the two peaks.
3.5 Chapter 3 Figures:

Figure 3.1 Loss of basal integrin results in increased oscillation amplitude of apically constricting amnioserosa cells. (a-b) Confocal z-stacks of sections of wildtype (a) and integrin null (mys−/−) (b) embryos undergoing DC stained for βPS-integrin (red), E-Cadherin (green) and DAPI (blue). Scale bars = 50µm. Integrins are basally localized in the AS (a”) and this localization is lost when integrin is genetically removed (b”). Scale bars = 10µm. (c) Representative cell in the AS undergoing apical cell oscillations. Cell outlines are marked by Cadherin:GFP, a representative cell trace is shown in red. (d) Period of cell oscillations are consistent regardless of background. (e) Amplitude of cell oscillations is increased in integrin null backgrounds (****p<0.0001). (f) Apical cell area is decreased in integrin null backgrounds (**p<0.01). (g) Mean cell radius of integrin null embryos is significantly smaller that both wildtype (****p<0.0001) and heterozygous controls (****p<0.0001). Heterozygous controls are slightly larger than wildtype (*p<0.05). Error bars indicate SEM.
Fig 3.2 Increased oscillation amplitudes correlated with decreased myosin speeds.
(a) Sample images from time lapse movies of cells in the AS with myosin (magenta) and E-cadherin (green) fluorescently tagged in heterozygous controls (a) and integrin mutant (b) embryos. Scale bars = 10µm (c) Schematic of apical cell oscillations (d) Sample cell output from PIV script of vector field generated. (e) Mean instantaneous speed of myosin, loss of integrin results in slower mean instantaneous speeds (**p<0.0001). (f-j) Myosin directionality is unperturbed in integrin mutants. (f) Rose diagrams of vectors angle for wildtype (f) heterozygous controls (f') and integrin null (f''). 180-0 represents
the medial-lateral direction in the AS. (g) Mean myosin intensity is unaffected by integrin loss. (h-i) x and y speeds of vectors are unperturbed in all the backgrounds studied. (j) Measurement of $|u|/|v|$ ratio shows that myosin flows are unpolarized in the AS, and integrin loss does not affect this polarization. Dotted grey line highlights a ratio of one, which indicates no direction. Error bars indicate SEM.
Fig 3.3 The subcellular localization and kinetics of E-cadherin based junctions is disrupted in integrin mutants. (a-b) Sample images of cell in the AS stained for E-cadherin (green) and F-actin (red) for control (a) and integrin null (b) embryos. Scale bars = 20µm (c-d) Representative graphs of fluorescence intensity along cell outlines for control (c) and integrin null (d) embryos. Pink dots marked peaks as counted by the software, teal lines represent the smoothed data, blue points represent actual data points,
and the blue line denotes the mean. (e) Mean fluorescence intensity of peaks. Integrin null cells peaks are more intense than controls (***p<0.001). (f) Maximum fluorescence intensity of peaks. Integrin null cells peaks have higher maximum intensity than controls (***p<0.001). (g) Number of intensity peaks per µm. Integrin null have less peaks per µm than controls (***p<0.001). (h-j) FRAP experiments on DE-cadGFP reveal that integrin mutants have different E-cadherin kinetics. (h) FRAP curves for control and integrin null embryos (i) Integrin mutants have a lower t1/2 than controls. (*p<0.05). (j) The mobile fraction of cadherin is not significantly different for integrin mutants.
Figure 3.4 Structure–Function mutations in integrin reveal a phenotypic variety of DC defects. (a-c) The penetrance of germ band retraction (GBR) (a), DC (b) and muscle attachment (c) were scored in integrin null embryos rescued with ubiquitously expressed mutant integrin constructs. Representative whole mount embryos for each of the rescue constructs stained with F-actin (red) and marked by integrinYFP are shown in e–k’, with F-actin in grey in (e’–k’). Scale bars = 50µm (d) Schematic of the integrin mutations used in this study (f–k) Integrin null embryos rescued with structure-function mutations were
scored for dorsal holes at stage 13-17. (f) Integrin null embryos have more dorsal holes in stage 15 than controls, indicating a delay. (g) Deactivating mutant integrinYFP*S196F results in a severe delays during DC. (h) A truncation of the cytoplasmic tail of integrin integrinYFP*804stop, results in a higher penetrance of defects, but no delay in DC. (i) A mutation reducing talin binding results in a less severe defect during DC. (j) A mutation in the hypothesized kindlin binding domain of integrin, integrinYFP*N840A results in a more delays, and more severe defects in DC than just integrin loss. (k) An activating mutation designed to increase talin binding, integrinYFP*YY>FF partially rescues delays associated with integrin loss.
Figure 3.5 IntegrinYFP*N840A does not affect apical cell oscillations and is not required for cell movement during DC. (a-c) Sequential overlay of cell outlines and centroid trajectory of IntegrinYFP*WT rescue (a), heterozygous control (b) and integrinYFP*N840A rescue (c) cells in the AS undergoing DC. Scale bar = 20 pixels. (d-e) Cell Movement is largely unaffected by integrin*N840A rescue. (d) Mean range of cell movement measured by ratio of trajectory area to cell area. (e) Mean instantaneous centroid speed normalized to cell area (f) Period of oscillation is unchanged for all backgrounds studied. (g) Amplitude of cell oscillation for integrinYFP*N840A is not significantly different from WT rescue, although WT rescue is higher than heterozygous controls (****p<0.0001). Error bars indicate SEM.
CHAPTER Four: Discussion

4.1 New Perspectives on Integrin Activation in Flies

Since the publication of our paper in 2014, our lab has continued to pursue a greater understanding of the mechanisms by which integrin regulation contributes to development. Specifically, utilizing FRAP analysis in combination with mathematical modeling, Hakonardottir, Lopez-Ceballos et al showed that the rate of adhesion assembly at MTJs was significantly increased when force was applied (Hakonardottir et al., 2015). Interestingly, they also demonstrated that when talin was unable to activate integrins, using the same talin*L334R transgenic described in our study, the increase in rate of assembly as a result of applied force no longer occurred. This suggests that although talin-mediated integrin activation does not play a significant role in early fly development, it may still be responsible for the reinforcement and stabilization of adhesion in a more nuanced manner, in addition to our identified role for integrin clustering. Currently, unpublished work in our lab aims to examine the role of outside-in activation on the regulation of turnover of integrin at MTJ’s (Lopez-Ceballos et al, personal communication). Using cations that have been shown to ectopically activate integrins in vitro, the authors show that outside-in integrin activation regulates integrin turnover to stabilize integrin adhesion and reinforce muscle architecture. This finding, taken together with our study (Ellis et al., 2014), and the work of others in Drosophila (Helsten et al., 2008) suggests that while outside-in activation plays key role in stabilizing Cell-ECM contacts in long-term, stable adhesions, talin mediated inside-out activation is not important in this system. In line with this hypothesis, we find that a point mutation in integrin designed to abrogate outside-in integrin activation,
integrinYFP*S196F, has a severe phenotype during development (Pines et al., 2011), this work, Figure 3.4). However this is contradicted from other work in our lab finding that other mutations that promote the extended conformation of integrins (L211) do not have severe phenotypes, suggesting that outside-in activation is not the only answer (Pines et al., 2011).

These findings provide interesting context to our study, because they suggest a more complex picture than we originally hypothesized in when Chapter Two was published in 2014. Integrin clustering clearly plays a key role in the reinforcement of adhesions; however, integrin activation additionally appears to be important for development, yet this function occurs independent of talin-mediated activation. Why would this difference occur in Drosophila? One possibility is that there are other inside-out integrin activators that play a more important role in inside-out integrin activation in Drosophila. Kindlin, for example, is known to mediate inside-out integrin activation (Calderwood et al., 2013; Harburger et al., 2009) (Iwamoto and Calderwood, 2015) and may therefore be a key player in mediating inside-out integrin activation in flies. Kindlin orthologs have been identified in Drosophila, and shown to play roles in myogenesis, but a full phenotype has not been published (Bai et al., 2008). In line with this, we find that an integrin structure function mutant defective in kindlin binding (Pines et al., 2011) (Chapter 3, integrin*N840A, Figure 3.3-3.5) has a severe phenotype during development, but does not perturb integrin behaviours associated with defective DC (Figure 3.5). Perhaps, in Drosophila, kindlin plays a crucial role in inside out activation, resulting in the severe phenotypes associated with the inability of kindlin to bind integrins. It is important to note, however, that it has not been established in Drosophila
that the integrin N840A mutations does abrogate kindlin binding, and this would be an interesting avenue for future study.

4.2 A Role for Rap1 Direct Binding to the Talin Head

In this thesis I also examined a potential role for Rap1-mediated recruitment of talin to the membrane. Although it has been well-established that Rap-1 in complex with RIAM can mediate talin recruitment to the membrane, a role for Rap-1 in mediating talin recruitment independently of RIAM is not yet well understood. Recent in vitro evidence has suggested that Rap-1 is able to bind talin directly, and therefore that RIAM may be dispensable for Rap-1 mediated talin recruitment. Specifically, NMR spectroscopy experiments demonstrated that Rap-1 was able to bind to the talin head in a GTP dependent manner (Goult et al., 2010). I therefore analyzed a point mutant in talin, talinGFP*K17E, which was contained within the domain hypothesized to bind to Rap1 by structural modelling (Goult et al., 2010). Although we did not find a dramatic observable phenotype, we did find that talinGFP*K17E was less stable at MTJs (Figure 2.1). This raises the possibility that Rap-1 can act alone to recruit talin to the membrane, but that this function is dispensable for development, perhaps because RIAM-mediated recruitment is more important. An interesting experiment to address this possibility would be to knock down RIAM from the muscles of the fly, which has been previously shown by our lab to result in an increase in talin turnover (Ellis et al., 2013), and see if this “sensitized” background alters K17E*talin localization to the membrane, and muscle development. Interestingly, a recent paper in Dictyostelium identified Rap1 as directly binding to the F0 domain of talin in this system, and further showed that inhibition of this
interaction, using a mutant in the homologous residue to that which we mutated (K16E) resulted in defects in adhesion-based morphogenetic events (Plak et al., 2016). We can envisage two possibilities for the role of RIAM independent recruitment of Rap1 to the membrane. Firstly, both integrin and RIAM are not present in unicellular Dictyostelium, suggesting that perhaps the ability of Rap1 to recruit talin to the membrane independently of RIAM is an evolutionary artefact. Moreover, previous analyses from our lab using RNAi knockdown of RIAM have suggested that RIAM loss does not have a strong phenotype in flies (Ellis et al., 2013). Perhaps when RIAM is lost, Rap-1 is able to compensate for this loss in flies specifically, and thus flies could represent the evolutionary turning point RIAM being necessary for talin recruitment to the membrane. Secondly, perhaps the requirement for Rap-1 independent requirement could be a tissue-specific requirement, such that different cellular contexts could utilize this system to recruit talin to the membrane. The follows with nuanced picture emerging of the complexity of integrin regulation during development, however, in our analyses we did not observe any tissue specific effects, so while we cannot rule this possibility out, there is little supporting evidence.

4.3 Integrins and Cadherins

Importantly, cadherins and integrins share many common characteristics, including: structural components, mechanosensitivity, connections to the actin cytoskeleton, and even some common signalling pathways (Weber et al., 2011). Given their functional similarities, and the requirement for coordinated adhesion for tissue integrity in driving dynamic cellular behaviours during morphogenesis, it is unsurprising
that crosstalk between integrin and cadherin has been well established in both *in vitro* and *in vivo* settings (Burute and Thery, 2012; McMillen and Holley, 2015; Weber et al., 2011). However, controversy still exists over precisely how cadherins and integrins modulate each other’s activity, and these interactions are likely context specific. For example, a 2010 study by Martinez-Rico et al (Martinez-Rico et al., 2010) found that in murine cancer S180C cells, more force was required to separate cell doublets when cells were seeded on ECM-coated beads compared to cells attached to poly-L-lysine coated beads. This suggests that engagement of integrin adhesion receptors specifically upregulates cadherin based cell-cell adhesion in this context. Importantly, this upregulation was found to be dependent on the actomyosin cytoskeleton (Martinez-Rico et al., 2010). By contrast, in 2011, Al-Kilani et al plated the same S180C cells on fibronectin micropatterns of varying sizes to modulate integrin adhesion, and then measured strength of cell-cell contacts using a cadherin coated bead. Interestingly, they found the opposite relationship; more integrin adhesion resulted in slower to form and less strong cell-cell adhesions (Al-Kilani et al., 2011). A number of technical explanations, such as 2D versus 3D cultures, methods of measuring strength of cell-cell adhesions, and stiffness of substrate (McMillen and Holley, 2015) could explain these opposing findings. Regardless, this discrepancy serves to highlight the complexity underlying integrin/cadherin crosstalk, and it is likely dependence on biophysical context. The importance of physical context to adhesion regulation highlights the importance of studying these interactions *in vivo*.

One *in vivo* study of particular relevance examines somite morphogenesis in zebrafish (Julich et al., 2015). During somite morphogenesis the surface of the zebrafish
paraxial mesoderm becomes coated in fibronectin as the tissue forms, while the core of the tissue does not form ECM. This localized ECM deposition depends upon active α5β1 integrin, which actively participates in the deposition of ECM in this case.

Interestingly, the authors found that this restricted ECM assembly on the exterior surface was caused by the formation of a complex containing both inactive integrin α5β1 and Cadherin 2 in adjacent cells within the interior of the paraxial mesoderm (Julich et al., 2015). Thus, they found that Cadherin 2 acts to maintain integrins in an inactive conformation in order to prevent ECM deposition on the interior surfaces of the somite.

Taken together with data from mouse keratinocytes demonstrating that cadherin-based adhesions act to localize traction forces to keratinocytes colony periphery (Mertz et al., 2013), a clear role for cadherin adhesions in cooperating with integrin adhesion to coordinate the transduction of force emerges.

Through what mechanism might integrins modulate the localization and turnover kinetics of cadherins during DC? One possible mechanism is that integrin signalling acts directly to modulate endocytosis of cadherins. In three-dimensional tissue culture, knockdown of integrin signalling via inhibition of focal adhesion kinase (FAK) and Src has been shown to increase cell-cell adhesion strength, and therefore decrease the migration of tumour cells (Canel et al., 2010). Furthermore, in line with our results, the authors use FRAP in their system to demonstrate that inhibition of integrin signalling decreases the half life ($t_{1/2}$) of cadherin on the membrane, and therefore increases cadherin turnover kinetics, which is precisely what we observe when we knockdown integrin (Figure 3.3I). Finally, the authors followed the endocytosis of biontinylated E-cadherin and utilized the endocytosis-inhibiting drug Dynasore to demonstrate that
Src/FAK acts on E-cadherin via the endocytic pathway, specifically by inhibiting internalization of E-cad. It is possible that our observations of defective cadherin localization, and increased $t_{1/2}$ of cadherin is similarly downstream of integrin signalling. FAK has been identified in *Drosophila*, and co-localizes with integrin, however, unlike in mice, it does not have a discernable integrin-dependent phenotype, and FAK mutants are viable and fertile (Grabbe et al., 2004; Maartens and Brown, 2015b). However, FAK overexpression in the fly muscles does result in a muscle detachment phenotype, similar to what has been observed for integrin loss, and therefore FAK could be an interesting candidate to consider in future studies of cadherin-integrin cross-talk. Two Src homologues have also been identified in *Drosophila*, Src42A and Src64b (Muda et al., 2002). Interestingly, although loss of a single Src homologue does not have a direct DC phenotype, genetic deletion of both homologues resulted in a DC phenotype, as a result of a failure in cell shape changes in the AS during DC (Tateno et al., 2000). Furthermore, Src42a is required for tracheal morphogenesis, and genetic deletion of Src in this system has been shown to modulate E-cadherin turnover (Shindo et al., 2008). It would therefore be interesting to genetically manipulate these downstream effectors of integrin signalling, and perhaps rescue or recapitulate our phenotype. This could distinguish between the signalling events that are modulating the interaction between cadherins and integrins and establish whether loss of integrins is upstream of changes in cadherin localization or vice versa.

An important caveat to these conclusions, however, is that we are unable to eliminate the possibility that loss of integrin perturbs apical-basal polarity, and this is what leads to the phenotypes that we observe. In *Drosophila* the Crumbs (Crb) protein
complex, along with cadherins, are a key component of epithelial cell polarity (Tepass, 2012). Interestingly, Flores-Benitez and Knust (2015) found that mutations in Crb resulted in defects during DC. Specifically, they found that the FERM domain of Crb was an essential negative regulator of actomyosin dynamics in the AS, and that Rho1 appeared to have a role in Crbs mediating actomyosin activity (Flores-Benitez and Knust, 2015). This raises the possibility the results presented here are not directly due to loss of integrin adhesion, but rather that defective cadherin localization results in loss of apical-basal polarity and thus perturbs actomyosin dynamics, or even more simply, that loss of integrin perturbs apical-basal polarity itself. However it is unclear whether these observed defects lie upstream of integrin loss or in a reciprocal feedback loop. Integrin is known to affect Rho1 signalling suggesting that integrin could play a part in this regulation (Calderwood et al., 2013). Furthermore, previous analyses using a constitutively active form of Rap1, another protein that affects apical basal polarity, has revealed significant DC defects, including in apical cell oscillations (Goodwin et al, personal communication), but no polarity defect based on Crumbs localization (data not shown). Moreover, our structure-function analysis of integrin adhesion during DC (Figure 3.4) demonstrates that integrin adhesion likely contributes through multiple pathways to DC, and therefore may influence DC through a variety of mechanisms.

4.4 Overall Conclusions

The two chapters presented in this work highlight the functional diversity of integrin adhesion receptors, even within the simplified system of *Drosophila*. The first chapter demonstrates a key role for integrins in long-term, stable adhesions that
characterize the embryonic and larval musculature of *Drosophila*. In this context, we find that talin-mediated integrin clustering is a key mechanism underlying the reinforcement of stable integrin adhesions during tissue growth and maintenance. By contrast, DC, a process we investigate in detail in Chapter Two, is characterized by short term, dynamic integrin adhesions. We find that during DC, integrin plays a role in modulating cadherin localization and kinetics, providing a putative mechanism for integrin’s effects on myosin flows. Interestingly, our clustering mutant *rhea*¹⁄₇ affects both types of integrin adhesions, as do many of the integrin mutants characterized in the second chapter of this thesis (Figure 3.4). This suggests that similar mechanisms underlie the regulation of integrin adhesions in both dynamic and stable adhesions throughout development. Furthermore, these mechanisms of regulation must be tightly controlled to maintain the appropriate stability of integrin-based adhesions, and to ensure that the development of complex, 3-D tissue architecture proceeds correctly.


