THE ROLE OF TALIN, A MASTER REGULATOR OF INTEGRIN-DEPENDENT MORPHOGENESIS, IN THE DROSOPHILA EMBRYO

by

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Abstract

The morphogenesis and maintenance of three-dimensional tissue architecture requires intricate, coordinated regulation of cell shape, position, and proliferation. Cell adhesion molecules, which mediate the attachment of cells both to one another (Cell-Cell adhesion) and to their surrounding extra-cellular matrix (Cell-ECM adhesion), are central regulators of morphogenesis during development and homeostasis. Integrins are the major family of cell-ECM adhesion receptors in metazoans and connect between ECM ligands and the actin cytoskeleton via an intracellular integrin adhesion complex (IAC). Integrins contribute to an impressive range of cellular processes and tissue behaviours, but the molecular mechanisms underlying diverse integrin function are not well described. In this thesis, I aim to shed light on this matter via a structure/function-based analysis of the core IAC component, Talin, in the context of *Drosophila melanogaster* embryogenesis.

Talin forms a direct structural link between integrins and the actin cytoskeleton and has been implicated as a regulator of integrin affinity for ECM ligands in the context of single cells in culture. My analysis reveals that in the context of a developing, intact organism, the principal functions of talin are to control IAC assembly, dynamics, and linkage to the cytoskeleton. Using a unique combination of forward and reverse genetics, live imaging, and immunofluorescence, I have uncovered novel insights into the role of talin as a regulator of integrin-mediated adhesion *in vivo*. I show that different domains of talin contribute to fine-tuning of integrin function in a tissue- and process-specific manner. The specific findings of this work are as follows: (1) two structurally distinct interactions between integrin and talin confer a developmentally regulated switch between modes of transient and stable adhesion (2) autoinhibitory control of talin provides a way to downregulate adhesion during morphogenesis.
and (3) the talin FERM domain primarily confers its effect on integrins through promoting receptor clustering and adhesion scaffolding rather than acting to increase integrin ligand affinity. In general, my work demonstrates how the interactions that talin makes within the integrin adhesion complex has major consequences for integrin function and thus for developmental control of tissue formation and maintenance.
Preface

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


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For this publication, I received help with molecular biology from Michael Fairchild and Mary Pines provided technical help with fly genetics, Western blots, qPCR, FRAP experiments, and sample preparation for imaging. I acquired all confocal images contained in this work, and performed all of the data analyses. Guy Tanentzapf and I designed experiments and co-wrote the paper.

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


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For this publication, I performed all experiments and analyzed all data with the exception of the biochemistry. NMR experiments (Figure 3.1) and vinculin binding assays (Figure 3.6) were carried out and analysed by Ben Goult (University of Leicester). Western blots shown in Figure 3.7 were performed by Jenny Long from the lab of Frieder Schock (McGill University). Michael Fairchild designed and made the Ubi-TalinGFP*E1777A construct and Nathan Harris, a graduate student in the lab of Mark Peifer (University of North Carolina), made the UASp-Rap1 constructs. Paolo Lobo from the lab of Filip van Petegem (UBC) assisted with homology modeling. Stefan Czerniecki provided technical assistance. Guy Tanentzapf and I designed experiments and co-wrote the paper.
Chapter Four: A version of this work is in preparation for publication.

Guy Tanentzapf and I conceived experiments and designed the project. Emily Lostchuck and Mary Pines assisted with fly genetics, sample preparation, image acquisition and FRAP. Michael Fairchild designed and made the *Ubi*-TalinGFP*L334R*, *Ubi*-headless-TalinGFP, and *UASp*-GFP-TalinHead*G340E constructs. Emily Lostchuck and I analyzed the data together. Mohamed Bouaouina, a research associate from the lab of David Calderwood (Yale University), performed and analysed the cell-culture based integrin-activation assays. Ben Goulart performed homology modeling and contributed the model shown in Figure 5. I wrote the preliminary version of the manuscript that is presented in this thesis.
# Table of Contents

Abstract ......................................................................................................................................... ii

Preface .......................................................................................................................................... iv

Table of Contents ......................................................................................................................... vi

List of Figures ............................................................................................................................... xii

List of Abbreviations ..................................................................................................................... xv

Acknowledgements ........................................................................................................................ xvii

CHAPTER ONE: Introduction .......................................................................................................... 1

1.1 Morphogenesis of Three-Dimensional Tissue Architecture ............................................... 1

1.2 Cell Adhesion and Morphogenesis ......................................................................................... 3
   1.2.1 Cell adhesion is required for morphogenesis. ................................................................. 3
   1.2.2 Cadherin-dependent cell-cell adhesion during morphogenesis. .................................... 4
   1.2.3 Integrin-dependent cell-ECM adhesion during morphogenesis. .................................... 6

1.3 Structure and Regulation of Integrin Activity ......................................................................... 8
   1.3.1 Structural control of integrin function ............................................................................. 8
   1.3.2 Integrin extracellular domains and ligand binding. ......................................................... 9
   1.3.3 Integrin transmembrane topology ................................................................................. 10
   1.3.4 The integrin cytoplasmic tails and inside-out activation. .............................................. 11
   1.3.5 Talin-induced integrin activation. .................................................................................... 12
   1.3.6 The role of kindlins in integrin activation. ..................................................................... 13
   1.3.7 Inhibitory interactions at integrin cytoplasmic tails. ...................................................... 14
   1.3.8 Integrin structure and activation: outstanding questions and controversies. ................ 15
1.4 The Structure and Function of Talin ................................................................. 18

1.4.1 The structure and major binding sites of talin. ............................................. 18
1.4.2 Regulation of talin activity. .............................................................................. 22
1.4.3 Recruitment of talin to adhesions. ................................................................. 23
1.4.4 The role of talin in mechanotransduction and cytoskeletal linkage. ............... 26
1.4.5 Talin in mammalian development and homeostasis ....................................... 29

1.5 Toward Understanding Tissue Morphogenesis: Integrin Function in Cell Culture Models of Cell Migration ............................................................................ 32

1.5.1 The integrin adhesome. ................................................................................... 32
1.5.2 Adhesion maturation during cell migration ..................................................... 34
1.5.3 Regulation of adhesion turnover. .................................................................... 35
1.5.4 The role of the microenvironment ................................................................. 36

1.6 Drosophila Models of Integrin-Dependent Morphogenesis ................................ 38

1.6.1 Integrins in Drosophila .................................................................................. 38
1.6.3 Early embryogenesis: gastrulation and germband extension. ......................... 42
1.6.4 Germband retraction ..................................................................................... 43
1.6.5 Dorsal closure ............................................................................................... 44
1.6.6 Muscle attachment ....................................................................................... 47
1.6.7 Other functions for integrins in Drosophila development ............................... 49

1.7 Aims and Scope of Thesis ................................................................................... 50

1.8 Chapter 1 Figures ............................................................................................... 52

CHAPTER TWO: Analysis of the Integrin-Binding Sites of Talin in Drosophila Development ........................................................................................................ 56

2.1 Introduction ....................................................................................................... 56

2.2 Results ............................................................................................................... 58
2.2.1 The conserved IBS domains act redundantly to recruit talin to sites of integrin-mediated adhesion downstream of integrin. ................................................................. 58
2.2.2 The IBS domains of talin are recruited to integrin by different mechanisms. .............. 61
2.2.3 Both IBS domains of talin contribute to talin function but have different roles during development. ........................................................................................................................................ 62
2.2.4 IBS-2 is required to maintain integrin linkage to talin and other IAC components. ........ 63
2.2.5 IBS-1 but not IBS-2 is required to maintain integrin linkage to the ECM. ....................... 66
2.2.6 Mutations in integrin that disrupt binding to talin phenocopy mutations in the talin IBS domains. ........................................................................................................................................ 66
2.2.7 IBS-1 and IBS-2 confer different, opposing roles in adhesion dynamics at the MTJ. ....... 68

2.3 Discussion ....................................................................................................................... 69

2.4 Materials and Methods ................................................................................................. 75
  2.4.1 Molecular biology. ...................................................................................................... 75
  2.4.2 Fly stocks and genetics. .............................................................................................. 76
  2.4.3 Immunohistochemistry, confocal microscopy, and image analysis. ...................... 77
  2.4.4 Western blot analysis ............................................................................................... 78
  2.4.5 qPCR ........................................................................................................................ 79
  2.4.6 FRAP ........................................................................................................................ 79

2.5 Chapter 2 Figures ........................................................................................................ 81

CHAPTER THREE: Rap1-Dependent Control of Talin Autoinhibition is Required for
Morphogenesis ....................................................................................................................... 100

3.1 Introduction .................................................................................................................... 100

3.2 Results .......................................................................................................................... 101
  3.2.1 Talin autoinhibition is a conserved mechanism between vertebrates and Drosophila. .... 101
3.2.2 Disrupting talin autoinhibition results in multiple morphogenetic phenotypes including delayed dorsal closure. ............................................................. 103

3.2.3 The talin autoinhibition mutant does not impinge on talin protein expression, stability, or sub-cellular localization............................................................................. 104

3.2.4 Impaired adhesion complex assembly does not underlie the phenotype conferred by the talin autoinhibition mutant........................................................................................................... 105

3.2.5 Blocking talin autoinhibition decreases talin turnover and promotes integrin adhesion complex assembly. .............................................................................................................. 106

3.2.6 The small GTPase Rap1 acts upstream of talin autoinhibition. ................................................ 106

3.2.7 The Drosophila RIAM homolog, pico, acts upstream of talin autoinhibition. .............. 108

3.3 Discussion .................................................................................................................. 110

3.4 Materials and Methods......................................................................................................... 113

3.4.1 Molecular biology and homology modeling................................................................. 113

3.4.2 Protein expression and purification. ............................................................................... 114

3.4.3 Vinculin binding............................................................................................................. 114

3.4.4 NMR spectroscopy. .................................................................................................... 115

3.4.5 Fly stocks and genetics. ............................................................................................... 115

3.4.6 Embryo staging and confocal immunofluorescence imaging........................................ 115

3.4.7 Embryo fixation and and image analysis...................................................................... 116

3.4.8 Time-lapse imaging. .................................................................................................. 118

3.4.9 FRAP ......................................................................................................................... 118

3.4.10 qPCR. ........................................................................................................................ 119

3.4.11 Western blots. ............................................................................................................ 119

3.5 Chapter 3 Figures ......................................................................................................... 121
CHAPTER FOUR: The Talin Head is Required for Adhesion Complex Assembly and Stability ................................................................. 134

4.1 Introduction .................................................................................................................................................................................. 134

4.2 Results ....................................................................................................................................................................................... 136

4.2.1 Integrin activation is not essential for fly embryogenesis .......................................................... 136

4.2.2 The talin head is required for integrin-dependent morphogenesis and muscle attachment 138

4.2.3 The talin head is required to stabilize the integrin adhesion complex at MTJs. ............ 139

4.2.4 rhea\textsuperscript{17} encodes a hypomorphic talin protein which disrupts talin head function ........ 140

4.2.5 The G340E mutation disrupts integrin clustering ................................................................. 142

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

................................................................................................................................................................................................. 142

4.3 Discussion .................................................................................................................................................................................... 143

4.4 Materials and Methods ................................................................................................................................................................. 148

4.4.1 Molecular biology .................................................................................................................................................................... 148

4.4.2 Fly stocks and genetics .............................................................................................................................................................. 148

4.4.3 Confocal immunofluorescence imaging and image analysis .................................................. 149

4.4.4 FRAP ...................................................................................................................................................................................... 150

4.5 Chapter 4 Figures .......................................................................................................................................................................... 151

CHAPTER FIVE: General Discussion and Conclusions ...................................................................................................................... 159

5.1 Overview of Findings ..................................................................................................................................................................... 159

5.2 Emergent Themes and Implications of The Thesis ..................................................................................................................... 160

5.2.1 Tissue-specific mechanisms define distinct modes of integrin function ............................ 160

5.2.2 Talin as a scaffold for adhesion complex organization and behavior ......................... 163

5.3. Limitations and Proposed Future Directions ............................................................................. 167

5.3.1 Reconciling differences between vertebrate and Drosophila integrins .......................... 167
5.3.2 How is integrin function regulated in the amnioserosa during dorsal closure? .................. 169
5.3.3 Understanding the role of too much adhesion during morphogenesis ......................... 171
5.3.4 Extension to mammalian systems and potential applications ....................................... 172

REFERENCES ........................................................................................................................................ 173

APPENDIX A: Novel, Tension-Responsive Adhesive Structures Mediate Cell Attachment to the ECM during Dorsal Closure ................................................................. 192

Appendix A Figures ................................................................................................................................ 192
List of Figures

Figure 1.1. Cell-Cell Adhesion and Cell-ECM Adhesion in metazoan cells. ..........................52

Figure 1.2. The domain structure of the integrin heterodimer and the structural basis for integrin activation in the transmembrane domain. .................................................................53

Figure 1.3. Cartoon schematic of talin. ..................................................................................54

Figure 1.4. Schematic of fly embryogenesis, stages 11-15. ..................................................55

Figure 2.1. Talin IBS-2 and the β-integrin cytoplasmic tail are conserved between Drosophila and humans. ..................................................................................................................81

Figure 2.2. Drosophila talin contains two conserved integrin binding sites that are each sufficient to localise talin to integrins at MTJs........................................................................82

Figure 2.3. Either IBS domain of talin is sufficient to localize talin to integrins at MTJs by independent mechanisms that depend on different parts of the β-integrin cytodomain....83

Figure 2.4. Expression levels of transgenic talin and βPS-integrin rescue constructs. .............84

Figure 2.5. Localization of fluorescently-tagged full length talin protein in the ectoderm and leading edge epidermis. ..................................................................................................................85

Figure 2.6. IBS-1 and IBS-2 mediate discrete subsets of talin function in development.......86

Figure 2.7. The pUbiTalin::EGFP transgene fully rescues embryonic defects in talin-deficient embryos. ..........................................................................................................................87

Figure 2.8. IBS-2 is required to maintain the connection between integrins and the IAC......89

Figure 2.9. Talin IBS-2 maintains the link between integrins and the IAC component PINCH. .................................................................................................................................91

Figure 2.10. Localisation of IAC components phospho-tyrosine and phospho-FAK in the Drosophila embryonic MTJ........................................................................................................92
Figure 2.11. IBS-1, but not IBS-2, is required to maintain the connection between integrin and ECM ligands.

Figure 2.12. Two distinct talin-binding sites in βPS-integrin are essential for fly development.

Figure 2.13. Mutations in each talin binding site of βPS do not disrupt localization of talin or paxillin but phenocopy talin IBS mutants.

Figure 2.14. Talin recruitment by βPS-integrin point mutants.

Figure 2.15. Mutations in IBS-1 and IBS-2 confer opposing effects on adhesion dynamics.

Figure 2.16. Proposed model of the functions of talin IBS-1 and IBS-2.

Figure 3.1. The mechanism of autoinhibition is structurally conserved between mammals and Drosophila.

Figure 3.2. Species alignment of domains required for talin autoinhibition.

Figure 3.3. Disruption of the autoinhibitory intramolecular interaction between the talin FERM and the talin rod leads to morphogenetic defects including delayed dorsal closure.

Figure 3.4. Talin recruitment and adhesion dynamics are altered by the E1777A autoinhibition mutation.

Figure 3.5. Talin autoinhibition is not required to maintain muscle attachment to the ECM or for IAC assembly at MTJs.

Figure 3.6. Talin autoinhibition does not affect vinculin recruitment to sites of integrin function in Drosophila.

Figure 3.7. Loss of FAK does not perturb the turnover dynamics of talinGFP or talinGFP*E1777A at MTJs.

Figure 3.8. Rap1 functions upstream of talin autoinhibition during morphogenesis.
Figure 3.9. Expression of Rap1-DN in the amnioserosa leads to defects in morphogenesis distinct from the phenotype of expression of Rap1-CA. .............................................................. 131

Figure 3.10. Pico/RIAM functions upstream of talin autoinhibition during morphogenesis. 132

Figure 4.1. Integrin-binding to the talin head, but not integrin activation, is required for muscle attachment....................................................................................................................................... 151

Figure 4.2. The talin head is essential for integrin function in Drosophila. ......................... 153

Figure 4.3. rhea17 encodes a missense allele of talin which severely disrupts talin head function......................................................................................................................................................... 155

Figure 4.4. The G340E disrupts adhesion complex maturation and integrin clustering. .... 157

Figure 4.5. Proposed model for G340 function in maintaining F2-F3 domain organization. 158

Figure A1. Focal Adhesion Like Structures are found on the basal surface of the amnioserosa during dorsal closure. ........................................................................................................................................... 192

Figure A2. FALS are developmentally regulated..................................................................................... 194

Figure A3. Evidence that FALS are sensitive to changes in both intracellular and externally applied forces......................................................................................................................................................... 195

Figure A4. Talin autoinhibition regulates FALS during dorsal closure. ............................. 196
List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AJ</td>
<td>Adherens Junction</td>
</tr>
<tr>
<td>ADMIDAS</td>
<td>Adjacent-MIDAS</td>
</tr>
<tr>
<td>AS</td>
<td>Amnioserosa</td>
</tr>
<tr>
<td>RGD</td>
<td>Arginine-Glycine-Aspartate</td>
</tr>
<tr>
<td>arm</td>
<td>armadillo</td>
</tr>
<tr>
<td>FERM</td>
<td>Band 4.1/Ezrin/Radixin/Moesin</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
</tr>
<tr>
<td>DFS</td>
<td>dominant female sterile</td>
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<tr>
<td>DC</td>
<td>dorsal closure</td>
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<tr>
<td>EM</td>
<td>electron microscopy</td>
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<td>ECM</td>
<td>extracellular matrix</td>
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<td>FRAP</td>
<td>Fluorescence Recovery After Photobleaching</td>
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<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FSC</td>
<td>follicle stem cell</td>
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<tr>
<td>FRET</td>
<td>Forster Resonance Energy Transfer</td>
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<tr>
<td>FRET-FLIM</td>
<td>FRET-Fluorescence-lifetime imaging microscopy</td>
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<td>GBE</td>
<td>germband extension</td>
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<td>germband retraction</td>
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<td>GLC</td>
<td>germline clones</td>
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<tr>
<td>GSC</td>
<td>germline stem cell</td>
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<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
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<tr>
<td>HUVEC</td>
<td>human vascular endothelial cell</td>
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<tr>
<td>if</td>
<td>inflated</td>
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<tr>
<td>IAC</td>
<td>integrin adhesion complex</td>
</tr>
<tr>
<td>IBS-1</td>
<td>Integrin Binding Site 1</td>
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<tr>
<td>IBS-2</td>
<td>Integrin Binding Site 2</td>
</tr>
<tr>
<td>ilk</td>
<td>integrin linked kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun N-terminus Kinase</td>
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<tr>
<td>LEE</td>
<td>leading edge epidermis</td>
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<tr>
<td>LDV</td>
<td>Leucine-Aspartate-Valine</td>
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<tr>
<td>MOP</td>
<td>membrane orientation patch</td>
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<td>MP</td>
<td>membrane proximal</td>
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<td>MIDAS</td>
<td>Metal-Ion-Dependent-A-Site</td>
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<td>misshapen</td>
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<tr>
<td>MEF</td>
<td>Mouse Embryonic Fibroblasts</td>
</tr>
<tr>
<td>mew</td>
<td>multiple edematous wings</td>
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<tr>
<td>mef2</td>
<td>myocyte enhancing factor 2</td>
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<tr>
<td>mys</td>
<td>myospheroid</td>
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<td>MTJ</td>
<td>myotendinous junction</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
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Particularly Interesting New Cysteine and Histidine Rich Protein

PALM
PhotoActivated Localization Microscopy

PSI
Plexin-Semaphorin-Integrin

PCR
polymerase chain reaction

PS
Position Specific

PIP2
PtdIns(4,5)P(2)

RIAM
Rap1-GTP Interacting Adaptor Protein

RNAi
RNA interference

scab
Scabrous

SHARPIN
SHank-Associated RH-domain Interacting Protein

siRNA
short interfering RNA

shg
Shotgun

SAXS
Small-Angle X-Ray Scattering

THATCH
Talin/HIP1R/Sla2p Actin-Tethering C-terminal Homology

Vh
Vinculin head

Vt
Vinculin tail

WT
Wildtype

YAP/TAZ
Yes-associated protein/Transcriptional coactivator with PDZ-binding motif
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Finally, the support of my incredible friends and family goes without saying. I love you all.
CHAPTER ONE: Introduction

1.1 Morphogenesis of Three-Dimensional Tissue Architecture

All metazoan life is initiated from a unicellular zygote, which, subsequent to fertilization, undergoes a complex program of cell growth, rearrangement, proliferation, and differentiation to establish a stereotyped body plan. The process through which three-dimensional tissue structure arises is called morphogenesis. The structure of various tissues and organs is essential to their function; therefore, identifying and understanding the conserved mechanisms that drive morphogenesis is of great importance to the fields of biology and medicine.

In an embryo, morphogenetic programs are executed starting from the oocyte where morphogens, the maternal determinants of polarity, specify what will become the major body axes of the animal. Deposition of morphogens is the key starting point from which morphogenesis of an embryo can begin. Subsequently, according to spatiotemporally regulated morphogen concentration gradients, morphogen-dependent signaling activates specific transcriptional programs that drive expression of genes that govern cell shape and position within a tissue [1]. Specifically, coordinated changes in cell behavior via cytoskeletal rearrangements and expression of cell adhesion molecules are the key aspects underlying morphogenesis [2].

Initiation of morphogenetic programs is a conserved phenomenon that is observed not only in during embryogenesis, but is also reiterated in many other physiological contexts. Morphogenetic processes are essential for tissue repair and maintenance; for example, wound
healing and formation of the milk-producing ductal network in the mammary epithelium both require activation of morphogenetic cell behaviors. Importantly, cellular behaviours and gene expression changes associated with morphogenesis must be carefully controlled to ensure homeostasis is maintained. Improper hijacking of morphogenetic mechanisms is therefore a common feature of many pathological conditions. Indeed, a hallmark of cancer progression and a key step in tumour dissemination is the upregulation of genes required for morphogenesis [3, 4]. Several regulators of morphogenesis have emerged as promising therapeutic targets to prevent metastasis [4, 5]. These discoveries have emphasized the importance of studying the cellular underpinnings of morphogenesis in model systems as these findings can be broadly applied to further our understanding of human health and disease.

In a developmental context, morphogenetic movements are observed throughout metazoan embryogenesis. A conserved aspect of early animal development is the transition from an undifferentiated sac of cells, called the blastula, to a more complex structure comprised of three distinct tissues called germ layers via a process known as gastrulation. Each germ layer will eventually give rise to differentiated tissues and organs in the mature organism. Following gastrulation, organogenesis begins through activation of tissue-specific morphogenetic developmental programs. Although there is great diversity across the animal kingdom with respect to how gastrulation and organogenesis occur to define each species’ unique body plan, several basic types of conserved morphogenetic cell behaviors dictate the transition from blastula to mature embryo in all animals.

The instructive role of mechanical forces in shaping developing tissue has been known for almost a century [6]. However, efforts to understand the contribution of biophysics to the emergence of tissue structure fell out of favour in the second half of the last century as the
molecular biology revolution took place and the focus of developmental biologists became understanding gene expression and signaling networks. Recently, with the advent of live imaging and the discovery that the biophysical attributes of the cellular microenvironment have important consequences for cell fate and survival [7-10], interest in defining morphogenesis from a mathematical, mechanical point of view has been rekindled. In the last several years, studies in *Drosophila* and *Xenopus* have revealed that cell shape change and differential changes in intracellular tension across a tissue can act as important cues dictating the progression of tissue morphogenesis and differentiation [11-17]. Understanding how mechanical signals are integrated with biochemical signals and transcriptional changes during morphogenesis is an emergent area of study, which will be necessary to complete our picture of how multicellular tissue architecture is established throughout the animal kingdom.

1.2 Cell Adhesion and Morphogenesis

1.2.1 Cell adhesion is required for morphogenesis.

Modulation of cellular positioning within a tissue is an inherent part of tissue formation. This is achieved through mechanisms of cell adhesion. In order to maintain their tissue architecture, developing tissues must withstand many intrinsic and extrinsic forces. Adhesion molecules are a key part of the molecular machinery that allow tissues to persist in the face of these forces and prevent dissociation of developing tissues into constituent single cells. However, cell motility is also an important aspect of animal development and morphogenetic tissue movements. Cell adhesion molecules also lie at the centre of the molecular machinery that governs cell migration; regulated assembly and disassembly of
various types of adhesive junctions is arguably the driving force underlying all morphogenetic movements [18]. An important outstanding question in the field of developmental biology is to elucidate how adhesion molecules are regulated during development to balance tissue integrity and plasticity, and how this balance is maintained throughout tissue homeostasis.

There are two broad classes of adhesion: (1) intercellular adhesion between adjacent cells and (2) adhesion between cells and their surroundings (Fig. 1.1). Disruption of either type of adhesion impinges on morphogenesis and maintenance of tissue integrity. Both types of cell adhesion are mediated by plasma-membrane-spanning adhesion receptors that bind to their extracellular ligands, and associate with intracellular adhesion complexes that link adhesion receptors to the cytoskeleton. Varying the composition of each adhesion complex can have great implications not only for how the adhesion complex attaches to the cytoskeleton, but also on cell-signaling events that occur in response to specific adhesive cues. Modulating the connection between adhesion receptors and the cytoskeleton can therefore play important roles in determining cell shape and behavior during tissue formation.

**1.2.2 Cadherin-dependent cell-cell adhesion during morphogenesis.**

There are many cell surface receptors that mediate cell-cell adhesion; the most widely expressed and best studied are the cadherins. Early ultrastructural studies on epithelial sheets described adherens junctions (AJs), electron-dense plaques where adjacent cells make contact with one another [19, 20]. Years later, calcium-dependent cell adhesion molecules, “cadherins,” were identified as core AJ components [20, 21], and are now well established to be the major family of transmembrane receptors mediating cell-cell adhesion (Fig. 1.1). To establish cell-cell contacts, cadherins cluster in the plasma membrane and make homophilic
adhesive contacts with cadherin molecules expressed on the surface of adjacent cells. Core cadherin adhesion complex components that localize to AJs and associate with cadherins include β-catenin, α-catenin and p120-catenin [22, 23]. Cadherin-mediated AJs are also essential for the establishment and maintenance of epithelial polarity, as their positioning within the plasma membrane plays key roles in setting up discrete apical and basolateral membrane compartments [22, 23].

Not surprisingly, early embryonic phenotypes have been identified in both mice and flies due to genetic loss of cadherin function. In the case of Drosophila, the widely expressed cadherin isoform, DE-Cadherin, is encoded by shotgun (shg). Loss-of-function analysis of shg in the early embryo was initially precluded because of a role for DE-cadherin in oocyte production [24, 25]. However, this problem was circumvented via a variety of genetic approaches including analyses of a strong shg hypomorph, and by simultaneous mutant analysis of the core AJ cytoplasmic component, armadillo (arm) [26]. Surprisingly, these analyses revealed that in Drosophila, epithelial structure is established in the absence of cadherin-mediated AJ function [26], but that early morphogenetic events of gastrulation require AJs to withstand forces that drive tissue invagination [11, 27]. In E-Cadherin knockout mouse embryos, the rounded cells of the early blastocyst do not undergo compaction of the pre-implantation embryo to form the polarized epithelium of the trophoectoderm [28, 29]. Taken together, analyses in both organisms confirm the conserved importance of cell-cell adhesion in mediating the earliest of morphogenetic events.
1.2.3 Integrin-dependent cell-ECM adhesion during morphogenesis.

The cellular microenvironment in which tissues are built is comprised of a gel-like protein substance called the extracellular matrix (ECM). During morphogenetic processes, cells must make and break connections with the ECM in order to form or modify three-dimensional tissue architecture. But how do cells attach to the ECM? In the late 1970s, Richard Hynes observed that the pattern of ECM fibril arrangement outside the cell and the organization of the actin cytoskeleton inside the cell closely related to one another and predicted the existence of a transmembrane adhesion receptor that would mechanically connect these two entities [30, 31]. His work, and the work of others, furthermore revealed that disrupting the structure and/or function of the ECM component fibronectin structure had severe consequences for cell shape, adhesion, and motility [31]. Work in model organisms extended upon these findings by using function-blocking antibodies to fibronectin to suggest that cell-ECM attachment is important in vivo for mediating morphogenetic processes during gastrulation [32]. Subsequently, identification of an additional antibody, “CSAT,” that could block the function of the putative ECM receptor led to work demonstrating that neural crest migration was impaired in chick embryos upon receptor loss-of-function [33]. However, the molecular identity of the ECM receptor itself remained elusive at this point. Biochemical approaches by multiple groups in various cell-based systems throughout the 1980s eventually clarified the structure and function of the integrin αβ heterodimer [31, 34, 35]. Integrins are now known to be expressed throughout metazoa and represent the principal family of cell-ECM adhesion receptors (Fig. 1.1).

Mammalian genomes encode 8 β-integrin genes and 18 α-integrin genes; together, the two subunits combine with one another to yield 24 observed αβ heterodimer combinations.
The diversity of integrins as well as the unexpected discovery that integrins were heterodimeric initially confounded direct genetic analysis of integrin function in vertebrate systems; however, elegant studies in the fly revealed that integrins are essential for the morphogenetic processes that shape the fly embryo ([36]; reviewed in detail in 1.6).

Functions for integrins have now been described in the development of most tissues in mammalian systems. Genetic ablation of β1-integrin, the most widely expressed integrin subunit, in the mouse revealed that integrin null embryos die at E5.5, following implantation [37]. The inner cell mass of the early embryo fails to differentiate properly and dies, presumably due to absence of integrin-mediated activities, which underlie morphogenetic changes in the presumptive endoderm and inner cell mass survival signaling at this developmental stage [37-39]. Mice deficient in laminin, the major component of the basement membrane at this stage, phenocopy the β1 phenotype lending further support to the hypothesis that cell-ECM adhesion between integrins and laminin are required for inner cell mass survival [40]. The finding that the very early stages of mouse development, including implantation, were not disrupted in the β1 knock-out mice was very surprising; it has therefore been suggested that maternally-contributed β1-integrin transcript and protein as well as contributions from other integrin heterodimers may mask earlier phenotypes but this hypothesis has not been rigourously tested [38]. Deletion of other integrin subunits, either throughout the whole organism, or in a tissue specific manner, has demonstrated requirements for integrins in many different morphogenetic processes that occur in the embryo including gastrulation [41, 42], neural tube closure [43], formation of the vasculature [44], skin development [45, 46], and blood cell function [38, 47, 48].
On the cellular level, integrins have been widely studied in the context of cell spreading and migration, two processes that are central to morphogenetic events. In order for a cell to migrate through the ECM, it must have some kind of cellular footing that allows it to make traction with ECM substrates and propel the cell body forward: integrins fulfill this role [49]. Integrins link to the actin cytoskeleton via an integrin adhesion complex (IAC) [49]. Hundreds of different cytoplasmic proteins can be found within the IAC depending on cell type and microenvironment [48, 50]. Collectively, the group of proteins that may reside within the IAC are known as the integrin adhesome [51]. Regulation of integrins during cell motility and the role of the integrin adhesome will be discussed in further detail in subsequent sections.

1.3 Structure and Regulation of Integrin Activity

1.3.1 Structural control of integrin function.

The importance of integrins in mediating morphogenetic changes is undisputed and the range of functions in which integrins have been implicated is astounding. How do integrins mediate such diverse roles? Detailed understanding of the structural and cellular regulation of integrins has provided some answers to this question.

Much effort has been put into elucidating the structure of integrins. The $\alpha$ and $\beta$ subunit associate with one another via non-covalent interactions, and together, may be roughly divided into three regions: (1) the extracellular domain (2) the transmembrane domain and the (3) cytoplasmic domain (Fig. 1.2a). Integrin heterodimers are unique in that they can exist in different conformations. It is generally understood that changes in receptor conformation have implications for integrin-ligand affinity. This phenomenon is referred to as integrin activation,
and is central to the ability of integrins to act as bi-directional signaling centres, able to integrate both extracellular and intracellular signals.

The delineation of what defines an active versus and inactive integrin has been a topic of much controversy. However, in general terms, when the extracellular domains of the α and β subunits are bent over one another, the integrin is thought to be inactive and have minimal ligand binding capacity. In contrast, when the α and β subunits are extended and the cytoplasmic and transmembrane domains are separated from each other, the receptor is typically thought of as active and primed to bind ligand [52]. The signal that induces integrin extension can come from either inside (effector binding to the cytoplasmic domain; “inside-out” signaling), or outside the cell (ligand binding to the ectodomain; “outside-in” signaling) [52]. The specific ways in which each integrin responds to signal to induce a conformational change has been the subject of much contention.

1.3.2 Integrin extracellular domains and ligand binding.

The α subunit is comprised of either 4 or 5 subdomains within its extracellular domain: a 7-bladed β-propellor domain, a thigh domain, 2 calf domains, and in the case of some α subunits, an “I” domain which can play important roles in regulating ligand affinity (Fig. 1.2a). The extracellular domain of the beta subunit is made up of seven subdomains: a beta “I” domain, a hybrid domain, a Plexin-Semaphorin-Integrin (PSI) domain, and four cysteine-rich EGF modules which serve to grant the β subunit its considerable conformational flexibility (Fig. 12a; [52]). The β-I domain contains three different cation coordination sites that are important for ligand binding [53]. The Metal-Ion-Dependent-A-Site (MIDAS) binds a Mg$^{2+}$ cation[54], the Adjacent-MIDAS (ADMIDAS) may bind both an activating Mn$^{2+}$ cation [55],
or an inhibitory Ca\(^{2+}\) cation [56], and the Synergistic Metal Binding Site (SyMBS) also binds a Ca\(^{2+}\) cation [56]. The canonical integrin-binding motif is the Arg-Gly-Asp (RGD) peptide sequence, which is found in many ECM ligands including fibronectin. The basic Arg residue fits into a cleft in the β-propellor of the α subunit and the acidic Asp coordinates with the Mg\(^{2+}\) in the MIDAS of the β-I domain. In the now widely accepted “switchblade” model of integrin extension, ligand binding is thought to trigger the downward movement of the β-I domain and subsequent 60° outward-swing of the β-hybrid domain [57, 58]. This induces the extension of the entire heterodimer and opening of the integrin head-piece [58]. Only about half the mammalian integrins recognize RGD ligands. For example, laminin binding to integrin occurs by a different, as of yet unclear mechanism. Additional integrin binding motifs have been indentified, including an Leu-Asp-Val (LDV) binding motif, which has been shown to coordinate MIDAS cations in a similar way to RGD [52, 59].

1.3.3 Integrin transmembrane topology.

The membrane spanning helices of both the α and β subunits make ionic interactions that stabilize the integrin heterodimer in the low affinity, bent confirmation. In this state, the β transmembrane domain is tilted relative to the α transmembrane domain, which is oriented perpendicular to the membrane [60]. This arrangement is maintained by electrostatic clasps between the α and β subunits at either side of the membrane. It has been proposed that disruption of this arrangement leads to separation of the two transmembrane domains, which subsequently leads to integrin activation [61]. It has been shown via flow cytometry that mutational disruption of these membrane clasps leads to increased integrin activation [60]. Therefore, it has been proposed that integrin heterodimer membrane topology and control of
tilt angle plays a key role in regulating integrin transmembrane signaling (Fig. 1.2b; [60, 62-64]). To provide an additional stabilization mechanism, the beta subunit contains a membrane-embedded basic lysine residue. The positively charged side-chain of this particular lysine “snorkels” towards the negatively charged cytoplasmic face of the membrane [63].

1.3.4 The integrin cytoplasmic tails and inside-out activation.

The cytoplasmic tails of integrins are fairly short, flexible, unstructured sequences. Whether the α and β subunits of a single integrin heterodimer associate with another in the cytoplasm is a point of contention. Initially, it was thought that cyto-domain separation occurred as a consequence of integrin activation. Specific mutations were identified in the α-integrin subunit that would promote integrin activation due to constitutive separation of the α and β cytoplasmic tails. However, it now appears, based on the work discussed above, that the significant separation event occurs at the level of the transmembrane domains of the heterodimer. Based on the relative flexibility and lack of structure of the cytoplasmic domains of both subunits, it is likely that they make multiple, transient low affinity interactions with one another.

Neither the α nor the β cytoplasmic domains have intrinsic enzymatic activity; however, together they provide an important scaffold for the docking of many proteins that are effectors of integrin signaling (reviewed in [65]). Most β-integrin subunits contain two conserved NPxY motifs, which are canonical binding sites of Phospho-Tyrosine-Binding (PTB) domain proteins. Many proteins have been identified that can bind to various β integrins, but the two best studied and best conserved examples are the talin head domain, which binds to the membrane-proximal NPxY motif, and kindlin, which binds to the
membrane-distal NPxY motif. Both proteins are notable for their roles in induction of inside-out integrin activation.

**1.3.5 Talin-induced integrin activation.**

Talin was discovered by Keith Burridge in the early 1980s [66]. It is a large, ~2450 amino acid protein comprised of an N-terminal FERM domain, known as the talin head, and a C-terminal rod domain, made up of several alpha-helical bundles [67]. How does talin head binding to β-integrin cytoplasmic tails promote integrin activation? Seminal work by David Calderwood and Mark Ginsberg demonstrated that the F2-F3 sub-domains of the talin FERM domain could bind β-integrin cytoplasmic tails, and that this binding event is essential for integrin activation [68-71]. Subsequent NMR and x-ray crystallography studies confirmed this interaction [72, 73]. Using a β3-integrin-PIPK1γ chimaera, it was shown that in addition to binding at the membrane proximal NPxY motif, the F3 domain of the talin head also makes interactions with the N-terminal-most part of the β-integrin cytoplasmic tail [73]. This “membrane-proximal” region of the β-tail forms an alpha helix that makes an extended interface with the talin head. When talin binds to integrin, a positively charged lysine residue (K322 in talin1) disrupts the salt-bridge that forms between inactive α and β integrin subunits at the inner edge of the membrane and facilitates the formation of a new salt bridge between K324 and a glutamate in β-integrin (D723 in β3-integrin) (Fig. 1.2b) [64, 73]. Mutation of this lysine abrogates integrin activation, although the talin head may still bind to integrin at the NPxY motif [73]. Further NMR and crystallography studies have shown that the talin head F3-integrin MP interaction is important for orienting a patch of positively charged residues on the face of the talin head F2 domain (Membrane Orientation Patch; MOP) towards the negatively-
charged PIP-enriched membrane [64]. Consistent with this finding, cell-based approaches using fluorescently tagged integrin point mutants showed that disrupting the $\alpha$--$\beta$ salt bridge and talin F2 membrane interactions are important for both integrin ligand binding and clustering [74]. It is now evident that formation of a salt bridge between integrin and talin, combined with talin-F2-membrane binding, induces a 20° change in tilt angle of the $\beta$-integrin transmembrane domain [62, 64, 75]. This conformational change is sufficient to induce integrin activation in the absence of applied external force or ligand binding [62, 71]. The structure and *in vivo* regulation of talin as it pertains to this thesis will be discussed in more detail in later sections.

### 1.3.6 The role of kindlins in integrin activation.

Kindlins are also a family of FERM domain containing proteins that bind the $\beta$-integrin cytoplasmic tail; however, they bind at the membrane distal NPxY motif [76] [77]. In the absence of talin, kindlins are not sufficient to activate integrins, but in some cell types, kindlin overexpression synergizes with talin to promote integrin activation [78]. Kindlins are therefore thought of as integrin “co-activators.” This effect appears to be dependent on the particular integrin isoform, as over-expression of kindlin has actually been shown to inhibit activation of $\beta$1-integrin [77]. While the mechanism of action is unclear, in most cases, knock-down or knock-out of kindlin attenuates integrin activation, clearly implicating a role for kindlin in this process. A recent study using single integrins embedded in membrane nanodiscs has suggested that the role of kindlins is to promote integrin clustering, and as such, to promote binding of activated integrins to multivalent ligands [79]. This is a somewhat contentious finding as the contribution of integrin clustering in regulation of integrin function
is disputed (see below in 1.3.7). *In vivo* studies on leukocytes illustrated that kindlin promotes adhesion stabilization during the leukocyte arrest that precedes extravasation [80]. This result may be explained by another report that, using flow cytometry and conformation-specific antibodies for LFA-1 (\(\alpha_\text{l}\beta_2\)), suggested that while talin is sufficient to induce integrin extension, kindlin is required to open the heterodimer head-piece and thus support strong adhesion to the vasculature during the transition between leukocyte rolling and leukocyte arrest [81]. An alternative explanation to these results, however, is that following talin-dependent integrin activation, kindlin simply acts to recruit other adhesion complex components. In line with this idea, early investigations on the worm kindlin homologue, *unc-112*, demonstrated that kindlin promotes adhesion complex assembly [82]. Therefore, it seems likely that in addition to increasing integrin avidity, kindlins also play important scaffolding roles, and may act as orchestrators of adhesion complex assembly, which in turn may lead to enhanced integrin activation.

### 1.3.7 Inhibitory interactions at integrin cytoplasmic tails.

There are several binding partners for integrin cytoplasmic tails that may act to inhibit integrin activation [83]. The group of Johanna Ivaska identified SHARPIN (SHank-Associated RH-domain Interacting Protein) in a siRNA screen for negative regulators of integrin activation [84]. SHARPIN binds to the membrane proximal region of the \(\alpha\)-integrin cytoplasmic tail and is thought to sterically inhibit the binding of both talin and kindlin to their respective binding sites on the \(\beta\) tail [84]. Many of the interactions that happen on the \(\beta\)-integrin cytoplasmic tail are regulated by Src-Family kinase-dependent phosphorylation of NPxY motifs. Talin is favoured to bind the membrane-proximal NPxY motif in the absence of
tyrosine phosphorylation [85, 86]. Other β-integrin binding proteins such as filamin and Dok1 can also outcompete talin for binding, and thus negatively regulate integrin activation [85-87]. A protein called ICAP-1 binds to the membrane distal NPxY motif of β1 integrin, thereby inhibiting kindlin binding and acting as an integrin inhibitor [88, 89]. Interestingly, the effect of ICAP-1 may be regulated by interactions with another cytoplasmic effector, KRIT-1, which binds ICAP-1, displacing it from β-integrin and essentially causing derepression of integrin activation [90]. The mechanisms governing the magnitude of integrin activity in vivo are only now beginning to become clear. The ability of the β tails to make differential interactions with binding partners in response to dynamic intracellular signaling and cues from the microenvironment will no doubt prove to be an important aspect of this regulation.

1.3.8 Integrin structure and activation: outstanding questions and controversies.

Whether integrins are bent or extended, and inactive or active, does not seem to be a question with a purely binary answer. Multiple lines of evidence from both crystallography and negative-stain EM studies have shown that there is a great range of conformations in which integrins can exist, and that the predominant conformation depends on many factors in any given cell. It has been shown that integrin heterodimers in the bent conformation can bind ligand [91, 92]. Therefore, it has been suggested that alternative to the integrin-extension model, more conservative conformational changes could take place to regulate integrin ligand-binding affinity [93]. Minimalistic experiments using single integrin heterodimers embedded in synthetic membrane bilayer nanodiscs showed that most integrins (~90%) are bent, but that twice the number of heterodimers can be induced to extend following the addition of factors that stimulate integrin activation (ie. addition of talin head domain or Mn++; [71]). This data
suggests that integrin extension plays at least some role in regulating integrin activity. Moreover, FRET-FLIM studies have shown that most integrins are bent outside of focal adhesions and extended within focal adhesions [94]. To reconcile differences between the various models, it has been suggested that the integrin heterodimer undergoes a “breathing” process whereby it wavers between conformational states during the transition from the closed to the extended position [53].

Most of the understanding of how integrins are regulated has come from investigations into regulation of integrin affinity and conformation. However, control of integrin avidity, or “clustering” is another level of integrin regulation that also merits some discussion. There are some reports that favour clustering over activation as the predominant mechanism governing integrin signal transduction [95, 96]. It is known that, in immune cells for example, the formation of concentrated microclusters of signaling receptors in restricted plasma membrane domains plays a significant role in signal amplification. In vitro studies demonstrated that α and β integrins could make homomeric interactions with one another to potentially drive integrin clustering and influence heterodimer separation [97]. However, evidence for these homomeric associations in vivo is currently lacking, and the predominant mindset of the field is still that control of integrin activity is achieved by ectodomain extension [52, 98]. Examination of integrin function in Drosophila cell lines established a model that was subsequently corroborated by studies in CHO cells proposing that talin-dependent integrin activation cannot be achieved unless in the presence of multivalent integrin ligands which drive integrin clustering [95, 99]. However, other studies clearly demonstrate that integrin activation can occur in the absence of clustering [71, 98]. Furthermore, in vivo studies in Drosophila imaginal wing discs demonstrated that integrin clustering could still be achieved in
the absence of the integrin-talin head interaction [100]. Resolution of these discrepancies remains an outstanding and contentious issue in the field. The relative contribution of clustering versus conformation to regulation of integrin activity is expected to vary according to cell-type, and integrin isoform.

The role of force, either from extracellular ligand binding, or actomyosin-dependent tugging on integrin cytoplasmic domains, in promoting integrin extension has also been disputed [53, 71]. It seems likely that there may be cell-type and integrin isoform-specific differences, thus contributing to the diversity of mechanisms that may regulate integrin function and adhesion. For example, it seems reasonable to postulate that leukocyte integrins should be sensitive to shear force, and this indeed is what has been discovered [53]. On the other hand, platelet integrins (αIIbβ3) do not seem to require force application to become active [71].

The focus of the field is shifting towards understanding how different integrin heterodimers mediate different functions. Such questions are directly relevant to the field of multicellular development and morphogenesis since the ability of slightly different integrin combinations to fulfill specific adhesive roles is one way by which integrin function can be developmentally regulated. Nonetheless, redundancy is an inevitable challenge, particularly in vertebrates where there can be over a dozen integrin heterodimers expressed in a single cell. Therefore, the importance of studying model organisms should continue to offer a unique window into understanding how integrins are regulated \textit{in vivo}.
1.4 The Structure and Function of Talin

1.4.1 The structure and major binding sites of talin.

Talin was discovered as a vinculin-binding protein that also labeled the end of actin microfilaments at putative sites of adhesion to the ECM. Thirty years later, and following decades of structural work using a combination of NMR, X-ray crystallography, Small-Angle X-Ray Scattering (SAXS) analysis, and electron microscopy, the full structure of the talin molecule has only recently been elucidated [101, 102].

Early negative stain EM images described talin as an elongated, flexible molecule that also was sometimes observed, by immunofluorescence, encircling actin filaments. A later study, also using negative EM of purified full length talin molecules, established the length of a full length talin molecule as approximately 50 nm and comprised of bead-like units [103]. Interestingly, the authors of this report further observed that in low salt conditions, this linear structure collapsed into a globular shape [103]. These important observations provided the first hints of the dynamic, multi-faceted roles talin plays in control of cell behavior.

The first sequence of talin cDNA revealed N-terminal homology to the band 4.1/Ezrin/Radixin/Moesin (FERM) family of proteins ([104]; at the time, band 4.1 and Ezrin were the only other known members). Common to talin and other FERM family proteins is the fulfillment of a structural role linking the plasma membrane to the cytoskeleton. A complete crystal structure of the talin head revealed the talin head assumes a novel extended conformation of 4 lobes: F0, F1, F2, and F3 (Fig. 1.3) [105]. This finding is in contrast to the organization of other FERM members where the domain is comprised of only three lobes that together take on a more globular structure. In addition, the talin head contains an unstructured
loop that extends from F1 [106]. It has been proposed that the linear arrangement of subdomains is to facilitate both integrin-binding at F3 as well as to maximize association between talin head and plasma membrane phospholipids [105, 107]. This arrangement would simultaneously promote integrin activation and stabilize recruitment of the talin head into integrin-mediated adhesions. The F1 loop contains positively charged residues that are predicted to further enhance binding to negatively charged phospholipids [106].

A series of structural studies revealed that the C-terminal ~2000 amino acids of talin (“the talin rod”) are composed of a series of bundles of 4-5 alpha helices, stabilized by hydrophobic cores and connected to one another by short unstructured regions [102, 108-114]. The structure of the domains was solved sequentially via NMR and crystallography over many years and thus it was a considerable challenge to establish how each of the domains fit together, and where the boundaries between each domain were in the context of the full length molecule. However, it has now been determined that the talin rod is comprised of 13 domains: R1-R13 (Fig. 1.3). R1 has 5 helices and R2-R4 have 4 helices; together, these 4 domains cluster together because the N- and C-termini of R2-R4 are all on the same side of the molecule. No lateral interactions between R1-R4 are observed. R5-R7 and R9-R13 are each made of bundles of 5 helices, and since the N- and C-termini of each bundle is located on opposite side of the molecule, these 8 domains are arranged linearly. R8 is a 4-helix bundle and does not disrupt the linearity of the rest of this part of the molecule because it extends from a loop in R7. This domain arrangement of talin is essential to the function of talin as both a force-bearing and force-transmitting molecule (discussed below in section 1.4.2 or 3).

There is also an integrin-binding site in the talin rod [114-116]. This domain is known as integrin-binding site 2 (IBS-2) in order to distinguish it from the integrin binding site in the
talin FERM domain (IBS-1; discussed in detail in 1.3.5). IBS-2 is not necessary or sufficient to induce integrin activation, although initial mutagenesis reports suggested that IBS-2 was required to link integrins to the cytoskeleton and promote adhesion complex assembly via binding to the membrane proximal helix of β3 integrin [114, 117, 118]. The studies examining IBS-2 structure and function are contentious because, with the exception of the study of Gingras et al [114], they were conducted using very small fragments of talin raising the possibility that the interactions observed with integrin are artifactual. Gingras and colleagues [114] identified a much larger region of the talin rod domain required for integrin binding and raised concerns about the physiological relevance of the other studies (ie. reports from Moes et al [117] and Rodius et al [118]). Furthermore, the initial immunofluorescence studies characterizing IBS-2 were conducted in CHO cells, which although useful for some assays, are not the most representative cell type in which to study the cell biology of integrins and the cytoskeleton. Super-resolution microscopy of focal adhesions in fixed cells revealed that talin is oriented with the head pointing towards the membrane and the rod extending into the cytoplasm to interact with vinculin and actin, and therefore precluding the talin rod from binding integrin [119]. Although it should be emphasized that these studies were carried out in only two cell types, in fixed cells, and in one type of adhesion, this observation further called into question the role of IBS-2 as a legitimate integrin-binding site. Nonetheless, a FRET-FLIM study in Mouse Embryonic Fibroblasts (MEFs) detected a stable interaction between β-integrin and IBS-2, but not between β-integrin and the talin head domain suggesting that the presence of a second integrin binding site in talin may confer a way to regulate the dynamics and stability of talin in adhesions [120]. The questions of the cellular function and regulation of IBS-2 are yet to be resolved.
At the very C-terminus of the talin molecule is the best-characterized domain through which talin can bind to actin; this domain is also important for talin dimerization and focal adhesion targeting [121, 122]. This particular actin-binding domain is a conserved Talin/HIP1R/Slp2p Actin-Tethering C-terminal Homology (THATCH) domain [113, 121, 122]. It is comprised of 6 helices; the first five helices comprise the core actin-binding domain and the sixth helix mediates talin dimerization [113]. Dimerization of talin monomers is achieved via the formation of an antiparallel coiled-coil between the dimerization helices of two talin molecules [113]. SAXS analysis revealed there is a range of possible conformations the talin homodimer may adopt ranging from parallel to tail-to-tail [113]. The conclusions of these analyses refuted earlier claims that talin predominantly existed as an anti-parallel dimer [123]. That arrangement would likely require an additional dimerization domain to be present in the talin head, which does not seem to be the case. Interestingly, the ability of the talin molecule to form dimers is essential for its actin binding capability: point-mutants designed to disrupt dimerization also exhibit markedly reduced actin binding [113]. The first helix of the THATCH domain negatively regulates actin binding [113, 124]. It is thought that stretch of the talin rod induced by actomyosin-dependent forces may be sufficient to displace this inhibitory effect although this has not been experimentally tested [113].

Many binding sites for vinculin have been identified along the length of the talin rod, as have multiple binding sites for Rap1-Interacting Adapter Molecule (RIAM; [102] [67]). There are also at least two additional actin-binding domains: one in the F2-F3 domain of the talin head [125] [126], and another in the rod domain, which has been mapped to region spanning between R4-R8 [125, 127].
1.4.2 Regulation of talin activity.

Many FERM proteins regulate their activity by making an autoinhibitory intramolecular molecular interaction between their FERM domains and another part of the molecule [128, 129]. For example, the actin-binding protein moesin uses autoinhibition to negatively regulate its actin binding activity [128]. Focal Adhesion Kinase (FAK) uses autoinhibition to negatively regulate its catalytic activity [129]. Though it has long been known that talin contained a FERM domain, and moreover despite observations of talin in both extended and globular conformations, the possibility that talin is regulated by autoinhibition had not been well explored until quite recently. Goksoy and colleagues used NMR to determine that indeed, the talin head interacts with the talin rod. Introduction of mutations that block this interaction resulted in increased integrin activation [130]. A subsequent study mapped the specific domains in the talin rod that are involved in this interaction and described a competitive relationship between talin head binding to the talin rod or binding to β-integrin cytoplasmic tails [108]. Elegant work using a combination of cryo-EM, SAXS, and modeling demonstrated that most of the talin in a cell is found in a compact, autoinhibited dimer. The two talin heads are located on the inside of the homodimer while the rod domains from both molecules form a doughnut that wraps around the outside of both head domains. In addition to the previously known interactions, this study identified many more intra- and inter-molecular interactions that would help to stabilize the globular structure of talin in its autoinhibited, dimerized state [101].

What are the consequences of talin autoinhibition? Cell fractionation experiments suggest that forcing talin into an “active,” extended conformation drives its localization into the membrane fraction. Cell biological approaches in HUVECs transfected with talin1 siRNA
and an autoinhibition mutant talin rescue transgene demonstrated that the effect of disrupting autoinhibition was to increase the number of focal adhesions that assembled in each cell [131]. Unlike other FERM domain proteins that are regulated by autoinhibition, it does not appear that relief of talin autoinhibition occurs via phosphorylation [132, 133]. A recent report suggests that talin autoinhibition is relieved by electrostatic interactions with the membrane. Negatively charged phospholipids attract the positively charged residues in the talin head that mediate the autoinhibition interaction with the talin rod, and thus can unclasp talin to promote its extension [133]. While this does seem a plausible mechanism, the recent cryo-EM structures [101] of talin call into question how the head might be able to gain access to the membrane, since it appears to be embedded in the middle of the doughnut structure that the autoinhibited dimer assumes.

1.4.3 Recruitment of talin to adhesions.

How is talin recruited to integrins? Several mechanisms have been identified. Most obvious is by binding integrins via either of the two integrin-binding sites in talin. This may happen at the plasma membrane, or earlier in the secretory pathway. However, there are many other ways that talin can be localized into adhesion complexes. Talin autoinhibition adds an additional regulatory layer that determines how and when talin localizes to adhesions. For example, are there autoinhibition-dependent and independent mechanisms of recruitment? Single particle tracking and PhotoActivated Localization Microscopy (PALM) were used to image single talin molecules in living cells and it was discovered that the isolated talin head constitutively labels the plasma membrane inner leaflet [134]. However, the full-length talin molecule was restricted to focal adhesions strongly suggesting that autoinhibition acts to limit
the access of the talin head to the membrane [134]. The authors of this study propose that most of the cytoplasmically localized talin is in the autoinhibited form, and that relief of talin autoinhibition occurs only within focal adhesions. Thus autoinhibition could provide a mechanism to prevent ectopic talin recruitment and integrin activation.

Mounting evidence suggests that membrane phospholipids play a dominant role in localizing and possibly stabilizing talin at focal adhesions [64, 134-136]. PIPK1γ binds to talin, and is recruited to focal adhesions to promote synthesis of PtdIns(4,5)P(2) (PIP2;[136]). Localized membrane domains of these negatively charged phospholipids are thought to be required to correctly orient the talin head relative to integrins and the plasma membrane to enable integrin activation [64]. Disruption of a PIP2 binding site in the talin head abrogates integrin activation [64]. Moreover, genetic ablation of PIPK1γ results in attenuated integrin activation, reduced talin recruitment and force-response defects suggesting that the ability of the cell to quickly form PIP2-rich membrane domains is an important factor in adhesion recruitment and maturation [136]. Interestingly, association with PIPKLγ and talin can promote polarized integrin secretion during directional cell migration, further illustrating the importance of membrane phospholipid composition in regulation of adhesion assembly and activity [137].

Activation of various signaling pathways also leads to recruitment of talin to adhesions. One possible route is via the formation of a complex between talin and RIAM and the small GTPase Rap1. Rap1 contains a membrane-localizing CAAX sequence at its C-terminus that could drive the recruitment of this tripartite complex to integrin adhesion complexes at the plasma membrane [138]. It has in fact been shown in cell culture that these three components contribute to integrin activation [139]. This phenomenon is dependent on
talin and RIAM binding, as well as the presence of the Rap1-CAAX. RIAM, as discussed above, binds to talin in several places.

It has also been proposed that vinculin plays an important role in localizing talin to adhesions. Autoinhibition-dependent masking of a vinculin binding site in the talin rod attenuates talin localization into the membrane fraction [140]. Via flow cytometry, it has been shown that vinculin-binding to talin is important for robust integrin activation, although whether this happens as result of enhanced talin recruitment, or some other mechanism that requires vinculin binding to talin has not been addressed [141]. Vinculin contains a talin binding head domain (Vh) and an actin binding tail domain (Vt) [142]. Vinculin exists in a closed conformation, whereby both Vh and Vt binding sites are masked. The process of vinculin opening – vinculin activation – requires talin, and the ability of vinculin to transmit force also depends on its talin binding capacity [142, 143]. It is therefore reasonable to suggest that localization of vinculin and talin are coordinately regulated, and positively feedback on one another.

Many lines of evidence have suggested that talin is a master regulator of adhesion complex assembly. Work in both cultured cells and in Drosophila have demonstrated that most adhesion components are not recruited in the absence of talin, and predict that integrin-talin complexes are the minimal units recruited to initiate adhesion complex assembly [100, 127]. However, one report has illustrated that the signaling molecule FAK may promote talin recruitment to nascent adhesions [144]. Interestingly, the authors of this study also showed that a talin-binding mutant β-integrin can still recruit talin in the presence of FAK [144]. Whether this is a cell-type or microenvironment-dependent phenomenon remains to be clarified.
Multiple molecular mechanisms lead to talin recruitment into adhesions. The fact that there are many pathways that seemingly fulfill the same function underscores two important facts: (1) talin is essential for adhesion complex function and (2) diverse means of recruiting talin to adhesions can provide a way to fine-tune adhesion function. Understanding how talin recruitment is regulated in different cell types and during different cell behaviors will inevitably provide important insights into how integrin function may be regulated in vivo.

1.4.4 The role of talin in mechanotransduction and cytoskeletal linkage.

In addition to biochemical mechanisms, talin can be recruited to adhesions in response to force; it is one of the best-characterized mechanosensitive proteins. Experiments using laser tweezers demonstrated that the force-dependent reinforcement of the link between fibronectin and the actin cytoskeleton is reliant on talin [145]. Furthermore, it was shown that the ability of adhesion complexes to mature in response to force, namely by increased recruitment of the talin-binding protein, vinculin, requires talin [146]. An explanation for this observation was provided by the demonstration that the talin rod unfolds in response to stretch to expose cryptic vinculin binding sites [147]. The length of talin in vitro is ~60nm; in vivo, the length of talin has been shown to vary between 5-8 times this length depending on subcellular localization and actomyosin contractility [148]. Altogether, this elegant body of work, which comes entirely from the lab of Michael Sheetz, suggests that talin acts as a spring that links between integrins and the actin cytoskeleton. As described above, it has also been predicted that actin binding at the talin THATCH domain is dependent on force-mediated unfolding of the talin C-terminus [113].
To date, relatively few reports have investigated the functional significance of talin’s mechanosensitivity in vivo. However, several studies in single cells have revealed some important clues about how mechanotransduction through talin, the actin cytoskeleton, and other adhesion associated molecules might contribute to cell migration (reviewed in [149]). Briefly, as a cell migrates, actin polymerization at the leading edge of a cell drives membrane extension. Leading edge protrusions must then be stabilized by adhesive contacts with the substrate that provide sufficient traction to move the cell body forward. During this process, the net flow of actin is backwards, away from the leading edge towards the cell body. This phenomenon is refered to as retrograde flow. Correlative fluorescence speckle microscopy was used to examine the movement of various adhesion proteins relative to F-actin retrograde flow during directed cell migration [150]. Interestingly, it was found that talin and vinculin exhibited variable trends in that sometimes they moved with the actin flow, while at other times, they appeared anchored to adhesions [150]. Based on this evidence, it was hypothesized that talin and vinculin form a molecular clutch that connects integrins to the cytoskeleton and provides traction for the cytoskeleton to pull on the adhesion and advance the cell body forward. However, with enough force (~2pN) imposed on individual talin molecules, the bond between talin and actin can slip and release; this idea explains a commonly observed effect known as adhesion slipping [145, 150]. Since vinculin also binds to F-actin, and is known to bear force in adhesions, the adhesion/F-actin link could be further reinforced upon force-dependent talin unfolding because this would facilitate more vinculin recruitment and linkage between the adhesion and F-actin [148, 151, 152]. Increased vinculin also has implications for biochemical signal conversion since vinculin activates the adhesion-associated signaling protein paxillin [146, 148, 152]. In further support of the molecular clutch hypothesis, it was
shown that in the complete absence of talin, fibroblasts could initiate cell-spreading events but they failed to establish ECM-cytoskeleton linkages and to make traction with the ECM [153]. Although integrin activation could be rescued by expression of the talin head, only expression of a full-length talin construct was sufficient to rescue cytoskeletal defects and the ability of cells to exert traction on the substrate [153]. This data is consistent with a model wherein the structure of the full-length talin contributes to its role as a mechanical, force-bearing link between focal adhesions and the cytoskeleton.

Talin mechanosensitivity also has consequences for how the adhesion complex assembles as it is now becoming clear that talin can orchestrate adhesion maturation in a stretch dependent manner. NMR studies showed that RIAM binding sites in the talin rod overlap with vinculin binding sites, and that the binding of RIAM and vinculin to talin is mutually exclusive [102]. This study predicted and provides some evidence in support of the hypothesis that when the talin rod is not stretched, it binds RIAM, but that under force, the talin rod extends and assumes a more linear conformation that favours vinculin binding [102]. This was corroborated by the observation that RIAM and vinculin are differentially localized within the cell: RIAM is present in nascent adhesions, whereas vinculin is present in more mature focal contacts [102]. Mutually exclusive binding to talin could furthermore be inferred from images of single adhesions where RIAM was observed enriched at the front of adhesions, and vinculin was enriched at the rear [141]. Time-lapse imaging further revealed the gradual displacement of RIAM by vinculin as adhesions mature [141]. The authors of this study propose there are two mechanisms for adhesion assembly and integrin activation that act independently of one another: a Rap1/RIAM-dependent mechanism and a vinculin-dependent mechanism [141].
1.4.5 Talin in mammalian development and homeostasis.

Analysis of the role of talin in mammalian development has been somewhat confounded by the fact that there are two isoforms of talin encoded by two different genes: Talin1 and Talin2. Talin1 and Talin2 are highly similar and maintain identical domain structure between one another [154]. Both isoforms are expressed in most tissues, but Talin2 has a more restricted expression pattern that is further complicated by a large number of possible splice variants [154, 155]. It has been shown in culture that Talin2 can compensate for loss of Talin1, even in tissues where Talin2 is not normally expressed [131, 153]. Talin1-null mice are embryonic lethal at embryonic day e8.5-9.5 due to gastrulation defects that occur as a result of impaired cell migration [156]. Surprisingly, talin null embryos implant normally but this presumably is due to maternal expression of talin, or compensation by Talin2 [156]. Talin2 knock-out mice are viable and fertile, although they exhibit a mild dystrophic phenotype, and reduced survival to adulthood was reported [157]. A tamoxifen-inducible Cre-based system was used to study the post-gastrulation effects of genetic ablation of Talin1 [158]. Angiogenesis was severely impaired and vascular lumen formation did not occur, although development of all other organs and tissues appeared to proceed normally [158]. Tissue-specific knock out of Talin1 in endothelial lineages gave rise to an identical phenotype in angiogenesis [158]. Talin2 is not expressed in the vasculature and therefore cannot compensate for loss of Talin1 in these cases.

The tissue-specific role of talin has been well studied in various types of blood cells as integrin-mediated adhesion plays various roles in hemostasis and immunity. Conditional knock-out of Talin1 in megakaryocytes, the precursors of platelets, resulted in severe hemorrhage and clotting phenotypes [159]. Surprisingly, the morphology of the cells appeared
normal, but platelet aggregation and adhesion was impaired [159]. A follow-up study used a megakaryocyte-specific knock-in of integrin activation-defective talin1 (L325R) to demonstrate the specific importance of integrin activation in the clotting process. Platelets expressing Talin1-L325R resembled Talin1 null platelets in that they also had defects in clot retraction and platelet adhesion [160]. However, the Talin1-L325R phenotype, but not the Talin1 null phenotype, could be rescued by treatment with MgCl₂, indicating that two factors govern efficient clot formation: (1) the presence of talin and (2) the ability of talin to activate integrins [160]. These studies provided the first direct example of the importance of integrin activation in a living organism.

Various groups have examined the effect of talin deletion in leukocytes. Controversially, it was discovered that leukocytes do not depend on integrin-mediated adhesion to migrate in three dimensions [161]. However, integrins and talin are required for dendritic cell extravasation out of the vasculature, an important step in mounting an immune response [161]. T- and B-lymphocytes are also important players in the adaptive immune response. Conditional ablation of talin in T-lymphocytes results in impaired immune synapse formation because T-cells are only able to make transient contact with antigen-presenting cells [162]. Moreover, cell polarization and cytoskeletal reorganization, which are required to form stable immune synapses, do not occur [162]. In B-lymphocytes, loss of talin disrupts homing from the spleen to the lymph nodes and/or the bone marrow [163]. Somewhat surprisingly, however, most aspects of B-cell development were rescued suggesting that talin-dependent integrin signaling does not play an important role in directing B-lymphocyte cell fate [163].

Talin also plays an important role in muscle function. Although both talin isoforms are important, Talin2 is the predominant isoform of talin expressed in the muscle. Conditional
knock-outs of Talin1 display only a mild muscle phenotype, with some defects in myotendinous junction maintenance [164]. Talin2 knock-outs give rise to a dystrophic muscle phenotype that is slightly more severe than that of Talin1 [157]. Recently, isoform-specific antibodies have shown that Talin1 and Talin2 are differentially localized to various muscle structures suggesting they fulfill different, specific roles in this tissue [165]. Both Talin1 and Talin2 were observed at myotendinous junctions, but costameres, the force-bearing structures where sarcomeres attach to the muscle cell membrane, only contained Talin2 [165]. Interestingly, it was established via biochemical approaches that Talin2 and β1D-integrin (the predominant muscle integrin) make a much higher affinity interaction than Talin1 and β1D-integrin, suggesting that Talin2 is specialized to support tissue integrity and adhesion in the face of the strong forces associated with muscle contraction [64]. To determine the effect of complete loss of talin in muscle, Talin1 was specifically deleted in the background of homozygous Talin2 knockouts [166]. This study demonstrated that despite continued presence of activated β1-integrin on the muscle cell surface, myoblast fusion and sarcomere assembly were severely impaired [166].

The role of talin has also been explored in the context of the specialized musculature of the heart. Talin2 is most highly expressed in the heart, whereas in healthy hearts, Talin1 expression levels are relatively low [154]. Interestingly, it was recently observed that Talin1 levels are elevated in failing hearts that have been subject to increased cardiac stress [167]. It was furthermore found that cardiac hypertrophy in response to prolonged heart stress could be attenuated by knock-down of Talin1 [167]. This finding has intriguing implications for the treatment of cardiomyopathy.
Ongoing studies will continue to define the tissue-specific roles of talin in both development and homeostasis. Although the presence of two talin isoforms considerably complicates the experimental approaches that must be undertaken, delineating the roles of Talin1 versus Talin2 in various tissues and developmental contexts also promises to be very interesting.

1.5 Towards Understanding Tissue Morphogenesis: Integrin Function in Cell Culture Models of Cell Migration

1.5.1 The integrin adhesome.

Focal adhesions have provided an important paradigm for studying integrin function in 2-D cell culture. A key regulatory aspect of integrin behavior has proven to be the composition of the integrin adhesion complex in different cell types and microenvironments. Combined approaches using bioinformatics, cell biology, and proteomics have revealed the composition of the integrin adhesome [51, 168-171]. Hundreds of proteins have been identified as part of a complex signaling network that forms the IAC between integrins and the actin cytoskeleton. The constituents of the integrin adhesome can roughly be divided into three categories: (1) proteins that bind integrins (e.g. kindlin); (2) proteins that bind actin (e.g. vinculin); (3) proteins that bind both integrin and actin (e.g. talin). The adhesome may be further divided into proteins that play a structural role versus proteins that play a role in integrin signaling; some proteins, such as FAK, act as both structural and signaling molecules (reviewed in [48]).

Proteomic approaches have demonstrated how the composition of the adhesome can change under different conditions. Treatment of cells with the myosin II inhibitor blebbistatin
considerably changes the composition of adhesions isolated for proteomic analysis; ~50% of the adhesome was demonstrated to be sensitive to myosinII activity [169, 171]. Interestingly, the localization of some proteins, including regulators of Rac activity, were found be negatively regulated by myosin II activity [171]. The discovery that adhesion composition was altered under different tensile conditions was not unexpected. In addition to talin, many proteins have been shown to display mechanosensitive characteristics: for example, zyxin relocalizes from focal adhesions to actin stress fibres in response to increased intracellular tension [172], and p130-Cas stretches, unmasking phosphorylation sites for Src [173]. Such tension-dependent events are predicted to have important consequences for downstream signaling and recruitment of other factors to the adhesion.

Differences in adhesome composition were also discovered upon plating cells on different substrates and ECM ligands [169, 170]. It has been estimated that each member of the adhesome is capable of making, on average, 8 different protein-protein interactions [51]. Therefore, it is not surprising that cues from both inside and outside the cell may contribute to alterations in these interactions, and thus change the overall adhesion composition and organization. Super-resolution studies have revealed an intriguing stratification of focal adhesions into functional layers of force-sensing proteins and signaling proteins [119]. Interesting outstanding questions remain with respect to the factors that determine adhesion complex stoichiometry and recruitment hierarchy, as well as spatial organization within individual focal adhesions.
1.5.2 Adhesion maturation during cell migration.

As a cell migrates, the adhesions must form, mature and strengthen, and then disassemble to allow advancement of the cell body. The process of adhesion maturation has therefore been a focus of intense study. During directional cell migration, dendritic actin networks drive membrane protrusion via the formation of a structure known as the lamellipodium. Nascent adhesions nucleate via unclear mechanisms, although integrin-ligand clustering and the dendritic actin network are both likely to contribute to adhesion formation (reviewed in [49]). Adhesion maturation occurs at a transition zone where the dendritic actin network of the lamellipodium is remodeled into linear actin bundles to form the lamella [174]. Nascent adhesions mature into focal contacts via myosinII-dependent mechanisms [175], and moreover, require a stress-fiber template [176]. In the lamella, focal contacts may mature into bone fide focal adhesions under the continued influence of actin retrograde flow-generated tension, which exerts a pulling force on adhesions and signals their maturation. As discussed in the previous section (see 1.4.4), a talin-dependent molecular clutch has been posited to facilitate progressive cycles of adhesion reinforcement and release that would likely contribute to adhesion maturation.

A mechanism for adhesion disassembly is essential to productive, directional cell migration. Actin-depolymerization is thought to be sufficient for the depolymerization of nascent adhesions. However, at the rear of the cell, adhesions are known to undergo a RhoA-dependent slipping event where they translocate further rearward [177]. This phenomenon is closely followed by adhesion disassembly. Calpain-cleavage of various adhesion proteins, including talin, has been implicated in this process, as well as RhoA-dependent generation of intracellular tension [178-180]. This disassembly mechanism is perhaps consistent with the
idea of integrins as participating in “catch” bonds, whereby as tension imposed on the integrin-ligand bond increases, the bond becomes stronger, up until a certain point, when too much tension causes the release of the bond [181].

1.5.3 Regulation of adhesion turnover.

Focal adhesion turnover can be governed by two factors: (1) the delivery and removal of integrin heterodimers to and from the plasma membrane and (2) exchange and recruitment of cytoplasmic factors to and from the adhesion complex. Recycling of integrins via clathrin-mediated endocytosis comprises an important mechanism to promote cell migration ([182]; reviewed in [183]). Many different signaling pathways have been shown to contribute to regulation of integrin receptor trafficking turnover. Specific mechanisms are likely to vary in a cell-type specific manner.

Extensive study of the molecular kinetics of various focal adhesion proteins has revealed that even in fairly long-lived adhesions, turnover of individual adhesion components happens very quickly [184, 185]. In general, it has been found that intracellular tension is a central factor in controlling the kinetics of adhesion turnover. Using photobleaching-based approaches, it was determined that the dynamics of structural proteins such as vinculin go down when intracellular tension is increased, and intriguingly, that the dynamics of some signaling proteins, such as paxillin, increase [186]. The opposite trends were observed when cells were treated with a myosinII inhibitor [186]. Interestingly, dynamics of some focal adhesion proteins have been shown to vary within single adhesions [184].

The ability of focal adhesions to undergo turnover and rapidly change their composition in the context of single cell migration in 2-D is of obvious paramount. However,
the ability of cell-ECM adhesions to modulate their dynamics and undergo junctional remodeling is also of critical importance for morphogenetic processes in the context of a tissue. It is clear that mechanisms that facilitate the remodeling and turnover of cadherin complexes is important for *Drosophila* embryogenesis [187, 188]. To date, very few studies have examined the contribution of regulated integrin turnover during tissue morphogenesis.

1.5.4 *The role of the microenvironment.*

It has been known for many years that the microenvironment plays a pivotal role in shaping tissues and determining cell fate [7]. The elasticity of the microenvironment has proven to be a master regulator of fate as mesenchymal stem cells cultured on the softest substrates commit to become neurogenic tissues, while mesenchymal stem cells cultured on stiff substrates commit to adopt an osteoblastic fate [8]. Work over the last two decades has suggested that this effect may be mediated through integrins. Pelham and Wang demonstrated that varying substrate stiffness could give rise to differential focal adhesion dynamics and morphology: adhesions on stiffer substrates were larger and less dynamic, whereas adhesions on softer substrates were more dynamic [189]. Furthermore, it was shown that by altering the distribution of cell-matrix contact, geometric control over the fate of a cell to proliferate or undergo apoptosis could be obtained [10]. The finding that cell shape and the geometry of cell-matrix contact sites played a role in cell fate determination was elaborated upon by the demonstration that mesenchymal stem cells committed to a bone cell lineage via a RhoA-dependent mechanism that was triggered in cells that had a spread morphology [9]. If cells were forced to assume a round shape, or if RhoA signaling was disrupted via expression of a dominant negative RhoA transgene, mesenchymal stem cells committed to become adipocytes
Other signaling pathways, including activation of YAP/TAZ responsive transcription programs, have been found to depend on substrate rigidity and cell shape control [190].

Since integrins have been implicated in environmental sensing, it seems reasonable to predict that they might play a role in the signaling process that ultimately feeds into cell fate control [191]. Integrins effectively “sense” the extracellular environment by binding to the ECM proteins that comprise the substratum. The density and identity of ECM ligands, as well as the particular integrin heterodimers expressed on the surface of any given cell, will influence how the cell interacts with its surroundings, and ultimately, will dictate the shape and cytoskeletal organization each cell assumes. Interestingly, it seems that density of receptor ligands provides a stronger influence over this sensory effect than total area of integrin-ECM contacts (reviewed in [191]). Integrins can also alter the microenvironment themselves by remodeling the fibrillar organization of various ECM components.

The differences in cell migration mechanisms in 2-D versus 3-D have become an area of great contention in recent years. In fact, the physiological relevance of focal adhesions and the great swath of biology that underpins their regulation have been called into question as it is not entirely clear whether focal adhesions form in 3-D tissue environments [192, 193]. It is conceivable that under the tensile conditions of a normal, physiological environment, focal adhesions are simply not induced to form [192]. Nonetheless, it is clear that focal adhesion proteins play a role in motility, even in a 3-D environment raising the possibility that adhesions are simply too small to see via conventional microscopy approaches [192, 193]. This particular issue brings to light the importance of studying integrins outside of cell culture models, in the context of living intact tissues.
1.6 *Drosophila* Models of Integrin-Dependent Morphogenesis

1.6.1 Integrins in *Drosophila*.

Although much mechanistic insight has gained from studying integrin-mediated adhesion in mammalian cell culture, these studies cannot recapitulate the complex tissue organization and microenvironment that is found in a living organism. Tractable genetics and live imaging technology developed over the past three decades have established *Drosophila* an invaluable model in which to study integrin function during morphogenesis and tissue maintenance in the context of an intact animal. Integrins in *Drosophila* are collectively known as the Position Specific (PS) integrins because αPS1 and αPS2, the first integrins to be identified in *Drosophila*, were initially described as a related family of surface glycoproteins that localized in distinct tissue compartments from one another [194, 195]. The obvious advantage of studying integrins in *Drosophila* is the decreased redundancy of the fly genome compared to mammals. For example, there are two β-integrin subunits, βPS (*myospheroid*) and βv [36, 196]. However, only βPS is widely expressed; detectable βv expression is limited to the developing midgut, further simplifying analysis of integrin function in most tissues [196]. The fly genome also encodes five α-integrin genes: αPS1 (*mew*; [197]), αPS2 (*inflated*; [198]), αPS3 (*scab*; [199]), αPS4, and αPS5, each of which displays a more restricted, and often non-overlapping, expression pattern. There is also only one gene encoding talin (*rhea* [200]; compared to 2 in vertebrates), and just two kindlin (*fermitin1/2*) genes (compared to 3 in vertebrates). This section will outline some of the best-studied features of *Drosophila*
development that rely on integrins, as they relate to this thesis, starting from the generation of an oocyte.

1.6.2 Drosophila gametogenesis.

The development of the fly embryo begins in the oocyte production factory: the ovary. Fly ovaries are paired; each ovary consists of 12-16 ovarioles [201]. The most anterior part of each ovariole is called the gerarium; the apical tip of each gerarium houses the germline stem cell (GSC) niche. A GSC undergoes an initial asymmetric division to retain one daughter cell as a GSC, and the other daughter is established as the cystoblast, which will eventually comprise a single developing egg [201]. The cystoblasts each undergo four rounds of cell division with incomplete cytokinesis to form a 16-cell germline cyst; one of these cells is specified as the oocyte [201]. The remaining germ cells in the cyst become support cells known as nurse cells that contribute maternal mRNAs and proteins to the oocyte [201]. Halfway along the length of the gerarium, the germline cyst encounters a population of somatic stem cells, the follicle stem cells (FSCs), that bud off, and according to poorly understood mechanisms, differentiate and proliferate into an epithelial monolayer that encapsulates the entire germline cyst [201]. Interestingly, integrin-mediated adhesion is required to support and maintain the laminin-containing microenvironment that comprises the FSC niche [202]. This cell-matrix contact is also thought to be important for regulating FSC proliferation [202]. Following encapsulation of the germline by follicle cells, the germline and the follicular epithelium are together referred to as single egg chambers. The follicular epithelium plays many key roles in control of germline development, establishing the body
axes of the oocyte, and generation of the eggshell that protects the developing zygote from the harsh environment.

The constituent cells of the follicular epithelium undergo many developmentally programmed morphogenetic changes that help to shape the developing oocyte (reviewed in [201]). Analyses of cell shape change and motility in this tissue have revealed a novel role for integrins in control of tissue elongation. Rotational movements of the entire follicular epithelium around the germline drive the elongation of the egg chamber from a spherical shape to a more ovoid shape. This process depends on integrin-dependent adhesion to the extracellular matrix [203]. Disruption of integrin function or of ECM assembly within the follicular epithelium leads to a “round-egg” phenotype; this defect was found to correlate with a cessation or misdirection of epithelial cell migration suggesting the rotation process is a vital part of the tissue elongation mechanism [203]. ECM fibers are basally secreted and polarize perpendicular to the anterior-posterior axis and the direction of tissue elongation of the egg-chamber and are thought to form a guideway for the migrating follicular epithelial sheet to rotate around the oocyte [203, 204]. Interestingly, whether or not clonal abrogation of integrin function was sufficient to disrupt rotation of the entire epithelium was dependent on the size of the integrin mutant clone: bigger clones halted the entire process, while smaller clones continued to migrate along side the WT tissue [203]. This suggests epithelial rotation happens as a result of coordinated migratory behavior across the entire epithelium. While it is clear that integrin-mediated adhesion is essential for egg-chamber development, a recent report provided an exciting demonstration of how too much integrin activity can also be deleterious. The Ste20 kinase Misshapen (Msn) functions to downregulate integrin levels at the rear of the cell so that it can retract and move forward with the advancing cell body [205]. In the absence of Msn
activity, integrin levels are not properly planar-polarized and cells remain stationary, thus halting migration and leading to a round egg phenotype [205].

Elongation of the egg-chamber continues after rotation of the follicular epithelium has stopped [201]. Integrin-mediated adhesion is required at later stages of egg-chamber morphogenesis to stabilize the basal actin cytoskeleton in follicle cells as they undergo a myosin-dependent oscillatory contractile behavior [206]. Cell oscillations are hypothesized to generate opposing forces that would resist the expansion of the rapidly growing germline encased by the follicle cells; no pulsatile contractions are observed at the anterior and posterior ends of the egg chamber [206]. Therefore, the authors of this study propose that tissue growth is constrained to the anterior and posterior ends of the egg chamber, thus giving rise to an elongated, ovoid tissue shape [206]. Perturbing integrin function led to altered pulsatile cell behaviours and defects in egg-chamber morphology [206].

Integrins are also required to support spermatogenesis in the testis. Mutant analyses of both integrin and talin null embryos revealed developmental defects in the architecture of the GSC niche [207]. A population of post-mitotic somatic cells known as hub cells defines the GSC niche in the testis. Integrin-mediated cell-ECM adhesion is required to anchor the hub cells at the apical tip of the Drosophila testis. GSCs surround the hub; this positioning determines the relative axis of cell division, an essential aspect of fate determination for stem cell daughters [208]. In the absence of integrin function, the hub cells are mispositioned and the preference for stem-cell division along the anterior-posterior axis is lost in the embryo [207]. Moreover, the ECM is disorganized indicating integrins are required to assemble ECM in this context [207]. In adult testes, loss of integrin function in the hub cells leads to loss of the entire stem cell niche, and effectively, the flies are sterile [207].
1.6.3 Early embryogenesis: gastrulation and germband extension.

Following oocyte fertilization, the *Drosophila* embryo undergoes 13 synchronous mitotic cycles with incomplete cytokinesis to establish the syncytial blastoderm. During this process, the nuclei migrate towards the surface of the embryo. At the end of the 14th division, plasma membrane extends between each of the ~6000 nuclei to form individualized cells; this event is known as cellularization. After cellularization, gastrulation occurs: the mesoderm invaginates internally at the ventral midline. Around this time, the endoderm is also specified and morphogenesis of various organ systems can subsequently begin, but the most visually striking feature of fly development that occurs at this time point is an axis elongation event called germband extension (GBE). The germband refers to two layers of ectodermal and mesodermal tissue that initially span the lateral length of the embryo, starting from just posterior to the head furrow and extending towards the posterior of the embryo (where the germ-cell precursors reside, thus the name “germband”). Through a remarkable process of actomyosin-driven cell intercalation and proliferation, the germband elongates to over twice its starting length. To accommodate this dramatic increase in tissue length, the germband extends dorsally and then back towards the anterior of the embryo, giving the impression the embryo has folded over on itself.

Phenotypes associated with loss of integrins during GBE have not been well described. However, integrin expression can be detected between the tissue layers of the mesoderm and the ectoderm following gastrulation [209]. Furthermore, there have been reports of tearing between these two tissue layers, as well as twisting of the germband, during the later phases of GBE in integrin mutant embryos [209]. Intriguingly, this phenotype did not seem to have any consequences for embryonic viability [209]. The role of integrins during GBE is likely worth
revisiting, as there is now an extensive body of work documenting what happens in the apical domain of the ectoderm cells undergoing GBE. Solving the mystery of how integrins might contribute to tissue remodeling and maintenance of tissue integrity during this process will no doubt prove to be very interesting.

1.6.4 Germband retraction.

Following the end of GBE, the tail of the embryo is positioned towards the anterior end of the body axis (Fig. 1.4A). Germband retraction (GBR) occurs to re-establish the tail at the posterior end of the embryo (Fig. 1.4B). GBR involves not only concerted migration and reorganization of the tissues that comprise the germband, but also implicates the amnioserosa (AS), an extraembryonic epithelium that initially sits in the gap between the ventral and dorsal extended germband. During the course of GBR, the AS cells must undergo many morphogenetic changes as they flatten and extend filopodia which mediate adhesion to the caudal germband ectoderm [210]. By the end of GBR, the AS forms the most dorsal tissue in the developing embryo and lies on top of the yolk sac. Failure of GBR is one of the most prominent phenotypes associated with embryonic loss of integrin function [209, 211]. αPS3βPS-integrin-dependent adhesion via a laminin-containing extracellular matrix is thought to mediate attachment between the amnioserosa and the caudal germband [211]. Genetic disruption of integrin or laminin function prevents the formation of lamellipodia that extend from the amnioserosa and facilitate the coherent movement of the AS and the germband [211]. Intriguingly, rescue of integrin function in the AS was sufficient to partially rescue GBR defects associated with complete lack of integrin illustrating how important the AS is during this process [211]. It will be interesting to elucidate what other roles integrins could have in the AS.
beyond lamellipodia formation. There is also no knowledge of what cellular behaviours integrins might be contributing to in the ectoderm. Clearly, there are many outstanding questions with regards to the activities of integrins during GBR.

1.6.5 Dorsal closure.

Following the completion of GBR, Dorsal Closure (DC) occurs to create a continuous layer of epidermis across the dorsal side of the embryo (Fig. 1.4C-E). Lateral epidermal sheets migrate over the AS, towards one another from opposite sides of the embryo; upon meeting one another, re-epithelization occurs. Both biochemical and mechanical signaling pathways have been identified that contribute to dorsal closure. Attention has largely been focused on the interplay between the front row of epidermal cells that make contact with AS, the Leading Edge Epidermis (LEE), and the AS. Over 60 genes are known to play a role in DC [212]. Many of these candidates are genes that are involved in patterning the embryo thus confounding their analysis via loss-of-function approaches. Furthermore, many mutations that are associated with failed DC display only partially penetrant DC phenotypes suggesting that there is built-in redundancy in the mechanisms driving DC. As a result, attempts to elucidate the developmental signaling pathways underlying DC have progressed quite slowly, but they have begun to illustrate the complicated crosstalk between multiple signaling pathways involved. In recent years, with the advent of live imaging, studies have focused on DC as a model to understand mechanical signaling during morphogenesis.

Multiple forces generated from within the tissue are now well established to be required for DC [213, 214]. In response to Jun N-terminus Kinase (JNK)-dependent signaling, a contractile actin cable assembles at the front of the LEE and spans the length of the interface
between the epidermis and the AS [215]. The actin cable is often thought of a purse string that helps to contract the hole and drive tissue closure [213]. A similar force-generating actin-based structure is also observed at the boundary of epithelial wounds [216, 217]. The leading edge epidermal cells form filopodia, which extend toward the dorsal midline and contribute to the migration of the epidermis over the AS. Once filopodia from opposite sides of the embryo are in proximity of one another, they make contact in a process known as zippering that is hypothesized to provide an additional pulling force for closure [218]. A further important part of zippering is segmental matching whereby signaling mechanisms are in place to ensure that the correct body segments from either side of the embryo line up with each other [218, 219]. Cell shape changes in the LEE also contribute to DC: they become elongated along the dorso-ventral axis and are presumed to impose an opposing tensile force to stabilize and balance the vectors of the other forces [214, 220].

Regulation of the actin cytoskeleton via the Rac and Rho small GTPases and their downstream effectors are well known to play important roles in DC [212]. Initial work focused on their role in assembly of the epidermal actin cable and filopodia, but more recently, the mechanical contribution of the actomyosin-dependent cell contractility in the AS has come to light. Studies describing the role of the non-muscle myosin II ortholog zipper in DC demonstrated that re-expression of Zipper only in the AS was sufficient to rescue the DC phenotype associated with loss of zipper [220]. Solon et al first reported pulsatile cell shape changes in the AS cells early in DC [14]. The period and amplitude of these cyclic cell shape changes becomes shorter as DC proceeds [14]. It was proposed that this behavior fulfilled a ratcheting function whereby sequential contraction of the AS helped to pull the dorsal hole closed; multiple mathematical models have corroborated this idea [14, 221]. Subsequent to this
study, two groups reported that cyclic assembly of actomyosin networks at the apical pole of AS cells corresponded with the observed cell cycles of cell shape change thus providing a mechanism to explain this phenomenon of reiterative tissue contraction [15, 16]. Cell extrusion also plays an important role in DC. About 20% of AS cells are extruded during DC [222]. It has been postulated that this may also constitute a force that contributes to DC although this is an unresolved and somewhat contentious question [222, 223]. At the completion of DC, upregulation of JNK signaling triggers AS involution into the interior of the embryo where cell death and engulfment by macrophages occurs via an unknown mechanism [224].

αPS3βPS integrins are required for DC; integrins are localized to the basolateral surface of both the epidermis and the amnioserosa [225]. Loss of integrin or laminin function leads to detachment of the amnioserosa from the underlying yolk, as well as disrupted AS tissue integrity and aberrant control of cell shape change [224, 225]. Analysis of βPS or αPS3 mutant embryos suggested possible defects in zippering in the absence of integrins [213, 225]; Gorfinkel and colleagues have suggested that this defect might manifest itself as a result of weakened adhesion between the epidermis and the AS, leading to misregulated AS contraction rather than any significant functional detriment to LEE filopodia [222]. Interestingly, integrins display a less restricted expression pattern in the AS and in fact are observed in the apical domain, a region from which integrins are usually excluded [226] [225]. One possibility is that integrins act as placeholders to precede the formation of septate junctions, junctional structures which are absent from the AS, but do form in the epithelium at the end of DC [226].

The role of integrins in triggering cell extrusion remains an interesting question in light of the mechanosenstivity of integrins and the fact that AS cell death occurs ectopically in the
absence of integrins [224]. The LIM-domain containing protein PINCH and its binding partner RSU-1 colocalize with integrins in the AS. In vitro data suggests that PINCH and RSU-1, in addition to playing roles in adhesion, might also antagonize JNK signaling in the amnioserosa [227]. It has also been demonstrated the JNK signaling positively regulates integrin expression during DC [215]. These findings raise interesting questions about the relationship between integrin and JNK-signaling since an upregulation in JNK signal normally happens in advance of AS cell death [224].

1.6.6 Muscle attachment.

The major β-integrin in Drosophila, βPS, is named myospheroid (mys) due to its lethal, highly penetrant muscle detachment phenotype. Alleles of mys were in fact isolated long before integrins were ever described [228, 229]. Muscle fusion and migration happens independent of integrin function [230]. Integrins are required, however, to form and maintain myotendinous junctions (MTJs), adhesive junctions where adjacent muscle cells attach to one another, as well as to specialized epidermal cells called tendon cells [231, 232]. MTJs form a mechanical linkage between the musculature and the skin and thus their integrity is of critical importance for locomotion. αPS2βPS integrins mediate muscle attachment; null mutations in either subunit result in embryonic lethal muscle detachment. Integrins are required for ECM assembly at the MTJ [232]. This ECM is known to contain many components including the Drosophila RGD-motif containing molecule, tiggrin, which has been established as a ligand for αPS2 integrins [233]. Laminin and thrombospondin are also present at MTJs [233, 234].

Integrins at the MTJ have emerged as an important in vivo model for understanding integrins. Comparisons have been drawn between the integrin-mediated adhesions that form at
MTJs and the integrin-containing focal adhesions that are observed in cell culture. The muscle cytoskeleton connects to αPS2βPS integrins at MTJs via the IAC; as with focal adhesions, talin is a core component. Similar to integrin mutations, loss of talin also leads to embryonic lethal muscle detachment in Drosophila, thus underscoring the vital role of talin in integrin function [200]. Inside-out signaling mechanisms recruit talin to integrins; the ability of integrins to cluster seems to be essential for this recruitment mechanism [235]. It is unclear whether integrin activation is a mechanism that is used in Drosophila tissues since experiments in cell culture revealed that activation of Drosophila integrins occurs independent of talin binding [99]. However, in vivo, rescue experiments of talin-deficient embryos using a ubiquitously expressed, full-length talin construct containing a mutation that abrogates talin head binding to integrin caused defective linkage between integrins and the ECM at MTJs [100]. These attachment defects could be rescued by expression of a constitutively-active αPS2 subunit [100]. The C-terminus of talin is also essential for integrin function in the embryonic musculature. Point mutations in full-length talin were designed to disrupt THATCH domain function, and thus to block the C-terminus from attaching to actin and/or dimerizing with other talin molecules. Expression of THATCH mutant constructs in embryos that lacked all endogenous talin resulted in severe muscle detachment phenotypes suggesting the ability of talin to make a direct link between the membrane and the cytoskeleton is essential for its function in maintenance of muscle adhesion [236].

Other proteins also mediate the link between integrins and the actin cytoskeleton including ILK (ilk) [237], PINCH (streamerduck) [238], paxillin (paxillin) [239], FAK (DFak56) [240], and tensin (blistery) [241]. The relative importance of each of these proteins in maintaining the link between integrins and actin, and in maintaining the integrity of MTJ
has been inferred from the severity of the loss of function phenotype of each of these genes. Surprisingly, some integrin-associated proteins, such as vinculin, do not have any phenotype upon genetic disruption despite a large body of research implicating mammalian vinculin as a critical regulator of integrin function [242].

Remarkably, it was recently shown using FRAP analyses that integrins at MTJs undergo turnover via clathrin-dependent endocytosis even though the adhesions that form at MTJs persist for several days following their initial establishment in the embryo [243]. It was further determined that this turnover is developmentally regulated: as the animal grows, and as the larvae hatches and muscle contraction occurs to drive locomotion, integrin turnover is downregulated, presumably to maintain tissue integrity in the face of increased tension imposed on the MTJs [243]. This hypothesis was strengthened by experiments using temperature-sensitive muscle contraction mutants. Upon induction of increased muscle contraction and intracellular tension, integrin turnover decreased, and upon induction of muscle relaxation, integrin dynamics increased [244]. These experiments provided the first in vivo evidence of integrin turnover during tissue remodeling.

1.6.7 Other functions for integrins in Drosophila development.

Integrins have characterized roles in many other processes that occur during Drosophila development. For example, many of the early insights into fly integrins were gained from studies in the Drosophila wing where integrin-mediated adhesions mediate attachment between epithelial cell layers. Integrins are also known to be involved in gut morphogenesis and migration [196], trachea development and lumen formation [245, 246], and blood cell migration [247]. I have limited my discussion here to just a few integrin-
dependent processes, which are intended to highlight the diversity of integrin functions in both stable and transient adhesive events.

1.7 Aims and Scope of Thesis

The aim of this thesis is to provide insight into the question of how diverse integrin function can be obtained during development. I focus my analysis and discussion on the linker protein talin since almost all integrin functions are dependent on talin. On the one hand, much is known about the structural and biochemical regulation of talin-dependent integrin functions. On the other hand, there is a great wealth of knowledge regarding Drosophila development and many descriptive studies have illustrated a role for integrins and talin in various contexts. However, mechanistic understanding of how integrins are regulated in the context of in vivo, developmental events is lacking. Thus, the goal of this work was to use a structure-function approach to provide specific insights into how integrin function is modulated by talin to elicit diverse cellular behaviours in a living, intact organism. Firstly, I explore how the ability of talin to interact with β-integrin through two distinct integrin-binding sites influences Drosophila embryogenesis (Chapter Two). Secondly, I investigate the significance of the ability of talin to autoinhibit itself as a regulator of integrin function (Chapter Three). Thirdly, I examine the role of the talin head in mediating Drosophila integrin function, and attempt to reconcile conflicting reports with respect to the importance of integrin affinity versus avidity regulation in Drosophila (Chapter Four). Overall, my studies strive to unify two divergent bodies of work on integrin-mediated adhesion and Drosophila development in order to add to
the understanding of how cell-ECM adhesion acts as a conserved driver of morphogenetic processes.
Typically, cadherin adhesion receptors make homophilic interactions with other cadherins expressed on the surface of adjacent cells to mediate cell-cell adhesion. The best studied example of a cadherin-mediated adhesion is the Adherens Junction, where apically localized cadherins between cells make extracellular interactions with one another. The intracellular domain of cadherin is coupled to the actin cytoskeleton through an adhesion complex that includes α-catenin, β-catenin, and p120-catenin. Cell-ECM adhesion is mediated by basally-localized integrin adhesion complexes that connect to the actin cytoskeleton through a large adhesion complex that varies according to cell type and context. In all cases, however, the linker protein talin is an essential core component of integrin-mediated adhesions.
Figure 1.2. The domain structure of the integrin heterodimer and the structural basis for integrin activation in the transmembrane domain.

(a) Major conserved domains of alpha-integrin (green) and beta-integrin (red). Together, the heterodimer may assume many different conformations. An extended conformation is shown here for ease of reference. Only some a-integrins contain “I” domains. The b-I domain contains cation coordination sites including the MIDAS, ADMIDAS, and SyMBS sites, which are important regulators of ligand affinity. (b) Electrostatic connections between the transmembrane domains of α- and β-integrin form clasps at both the outer-membrane and inner-membrane periphery that help to stabilize the ab heterodimer in an inactive conformation. During the process of inside-out integrin activation, talin head binding at the inner-membrane interface induces unclasping of integrin transmembrane interactions. This results in separation of the α- and β-transmembrane domains and triggers a conformational change across the entire heterodimer that ultimately results in increased affinity of the integrin heterodimer for its ligands.
Talin is comprised of an N-terminal FERM domain made up of four lobes (F0, F1, F2, and F3), and the C-terminus is comprised of 13 bundles of 4-5 alpha helices (R1 - R13). The position of the major protein-protein interacting domains that are relevant to this thesis are colour-coded as indicated in the legend.
Figure 1.4. Schematic of fly embryogenesis, stages 11-15.
(a) Stage 11: The embryo has completed GBE and the AS is flattened between the head and the tail of the embryo in preparation of GBR. (b) Stage 12: During GBR, AS cells become flatter and more spread. (c) Stage 13: The AS covers the dorsal surface of the embryo. At the onset of DC, an actin-rich cable is observed at the epidermis-AS interface (inset in green box in c’) and the epidermis begins migration over the AS. Arrow in c’’ indicates direction of migration. (d) Stage 14: midway through DC, AS cells are apically constricted and elongated along their apical-basal axis. (e) Stage 15: DC is completed and the AS has been degraded. The yolk is now wrapped in the fused midgut. A temporary scar is visible on the dorsal epidermis as inter-cellular junctions are reformed. Colours correspond to tissues as indicated. Asterisk indicates the position of the germband. Anterior is to the left in a-e, c-e’. Dorsal is up in a-e, c’’-e’’.
CHAPTER TWO: Analysis of the Integrin-Binding Sites of Talin in *Drosophila* Development

2.1 Introduction

Adhesion receptors play many roles throughout animal development. During dynamic morphogenetic events, transient Cell-Cell and Cell-ECM contacts provide traction for cell migration and facilitate cell rearrangements [248]. Once three-dimensional tissue architecture is established, it is maintained by stable-long lasting adhesion. Diverse types of cell adhesion may be obtained through the use of different adhesion receptors, or by utilising the same adhesion receptor but changing the composition of the intracellular complex with which it associates. For example, integrins - the principal mediators of cell-ECM attachment in metazoans - associate with different intracellular components over the lifetime of focal adhesions [191], a process referred to as “maturation.” This can profoundly affect how the integrin adhesion complex behaves. For instance, integrin binding to different cytoplasmic adapter proteins can act to switch on and off dynamic adhesive processes associated with cell migration [249].

The large cytoplasmic adapter protein talin provides an essential link between integrins and the cytoskeleton by directly binding actin [236] and by recruiting downstream components of the adhesion complex [100, 146]. In addition, talin plays an important role in regulating the affinity of integrins for their ECM ligands, a process known as inside-out activation [61, 70]. Talin contains two known integrin-binding sites (IBSs): IBS-1 is located in the N-terminal end of the protein, while IBS-2 is in the C-terminus. The IBS-1 domain of talin has been studied extensively for its ability to confer conformational changes in the integrin molecule.
resulting in inside-out activation. Structural studies of IBS-1 have revealed key residues essential for the binding of talin IBS-1 to the integrin cytoplasmic tail [64, 69, 72, 73]. In vitro and in vivo studies have further shown that the binding of talin IBS-1 to the membrane proximal NPxY sequence in the integrin cytoplasmic tail is required for integrin activation [69, 70, 73]; [100]. The crystal structure of talin’s IBS-2 domain has also been resolved and residues have been identified in both talin and integrin that mediate their interaction [114, 117, 118, 250]. Importantly, IBS-2 was shown to interact with a membrane-proximal part of the integrin cytoplasmic tail, distinct from the NPxY motif; this interaction does not induce inside-out activation [114, 116-118]. However, to date the roles of the IBS-2 domain of talin have not yet been explored in the context of a whole developing organism.

We previously showed that targeted disruption of the talin IBS-1 domain does not completely abrogate talin function but leads to a specific defect: detachment of integrins from the ECM late in embryonic development [100]. This suggests that the talin IBS-2 domain may be partially redundant with the IBS-1 domain and also raises the possibility that each IBS can have different and specialized roles during development. In this study we extend our previous analyses to address the specific developmental roles of IBS-2. Our results confirmed that IBS-1 and IBS-2 act redundantly in some contexts but mediated specialized functions over the course of development. In particular, we observed that IBS-1 had a more pronounced role in linking integrins to the ECM, while IBS-2 was more important for linking integrins to the intracellular integrin adhesion complex. Consequently, we propose that developmental processes that depend on ECM or cytoskeletal linkage were more sensitive to disruption in the IBS-1 or the IBS-2 domains of talin, respectively. Our work defines the relative importance of
each IBS domain of talin during development and provides insight into how the functional
diversity of integrin-mediated adhesion is attained.

2.2 Results

2.2.1 The conserved IBS domains act redundantly to recruit talin to sites of integrin-
mediated adhesion downstream of integrin.

The IBS-2 domain of talin is comprised of two bundles of five helices: IBS-2A (residues 1983-2130 in fly talin) and IBS-2B (residues 2131-2297 in fly talin). While the entire IBS-2 domain is well conserved between fly and vertebrate talin (66% sequence similarity, 47% sequence identity; Fig. 2.1A) IBS-2A is much more conserved (80% similarity, 71% identity) than IBS-2B (57% similarity, 41% identity). Our research group previously expressed a protein fragment corresponding to the IBS-2A domain of fly talin showing that it was stable, soluble and readily crystallized, thus allowing its structure to be elucidated [250]. We tagged the IBS-2A domain with GFP (UAS-GFP-IBS2, Fig. 2.2A), ectopically expressed it in muscles and studied its localisation. In *Drosophila* embryos, the major integrin-containing adhesive junctions are formed at MTJs, where muscle ends attach via the ECM to the epidermis. We have previously developed a robust assay to measure the relative enrichment of proteins at MTJs by quantifying the signal at the MTJ and expressing it as a ratio over the signal in the cytoplasm [236] [100] [235]. Talin is known to specifically localise to MTJs [200] [100]. When the tagged IBS-2 domain (UAS-GFP-IBS2) was expressed in muscles using the dMef2-GAL4 driver, it showed a two-fold enrichment at MTJs compared to the cytoplasm (Fig. 2.2B; Fig. 2.3A). This localisation was dependent on integrin since in the absence of βPS-integrin, UAS-IBS2-GFP was not enriched at MTJs. (Fig. 2.1B; Fig. 2.3C). Previous in vitro studies identified two sets of mutations, K2085>D/K2089>D and
L2094>A/I2095>A in vertebrate talin (equivalent to K2094>D/S2098>D and L2103>A/I2104>A in fly talin, respectively) that disrupted the binding of IBS-2 to the integrin cytoplasmic tail [117, 118]. The K2094>D/S2098D residues were mapped to a position on the surface of the IBS-2 domain, where the L2103>A/I2204A residues were mapped to be in location buried within the structure of the IBS-2 domain. We chose to examine both sets of mutations in fly talin in order to differentiate the specific effects of disrupting IBS-2 function from other potential off-target effects induced by each set of mutations. We introduced both these sets of mutations into UAS-GFP-IBS2 and found that the mutated IBS-2 domain was not enriched at MTJs, regardless of whether the L2103>A/I2104A mutation or the K2094>d/S2098D mutation was present (Fig. 2.2B; Fig 2.3E, G). Similarly, and consistent with our published data, when the IBS-1 domain was tagged with GFP (UAS-IBS1-GFP) and ectopically expressed in muscles using the dMef2-GAL4 driver, it localised to MTJs in an integrin-dependent fashion [[100]; Fig. 2.2A, B; Fig. 2.3B, D]. Moreover, introduction of a mutation into UAS-GFP-IBS1 designed to disrupt its interaction with the integrin cytoplasmic tail (R367A; equivalent to R358A in vertebrate talin; (see [72]) abolished the enrichment of the tagged IBS-1 domain at the MTJs (Fig. 2.2B; Fig. 2.3F). Also in accordance with our previous studies, we observed enrichment of UAS-IBS1-GFP in the nucleus. Our prior work strongly suggests that this nuclear staining is unlikely to be functionally significant and is rather an artifact of overexpression of the GFP-tagged fusion protein [235].

To further assess the importance of each IBS for talin localisation, mutations designed to disrupt the talin IBS domains were introduced into full-length talin. We utilized a previously established protocol and introduced point mutations into a ubiquitously expressed full-length talin rescue construct [[100]; see Materials and Methods]. Five GFP-tagged rescue
constructs were made: wildtype talin-GFP, IBS-1 mutant (talin R367A), IBS-2 K2094>D/S2098>D mutant (talin KS>DD), IBS-2 L2103>A/L2104>A mutant (talin LI>AA), and IBS-1/IBS-2 double mutant (talin R367A; LI>AA). We ensured that our rescue constructs were expressed at similar levels to the wildtype rescue construct (Fig. 2.4A). We measured the relative enrichment of the full-length GFP-tagged talin transgene at the MTJ by determining the ratio of MTJ signal to cytoplasmic signal. The wildtype talin-GFP was enriched at MTJs, similar to the distribution of the endogenous protein [200] [100], as GFP signal at the MTJs was over 4 times greater than in the cytoplasm in a wildtype background (Fig. 2.2C,E). In comparison, the IBS-1 mutant talin R367A and the two IBS-2 mutants, talin LI>AA and talin KS>DD, showed reduced enrichment at MTJs (Fig. 2C, G, I, K). Moreover, the IBS-1/IBS-2 double mutant R367A; LI>AA was not enriched at MTJs in a wildtype background, as the ratio of MTJ to cytoplasmic signal was approximately 1:1 (Fig. 2.2C, M). This result is consistent with previous data [100, 235] suggesting that in a wildtype background, ectopically supplied talin competes with endogenous talin for integrin binding sites. Moreover, GFP-tagged full-length rescue constructs containing mutations in either the IBS-1 or IBS-2 domain compete less efficiently with the endogenous protein when compared to the wildtype full-length rescue construct.

As endogenous talin can compete with the GFP-tagged full-length talin rescue constructs, we studied their localisation in talin-deficient embryos (see Materials and Methods). In the absence of endogenous talin, there was no significant difference in enrichment at MTJs between the GFP-tagged full-length wildtype talin rescue construct and GFP-tagged full-length rescue constructs containing mutations in either the IBS-1 or IBS-2 domains (Fig. 2.2D, F, H, J, L). However, the enrichment of the double IBS mutant talin was
reduced by a statistically significant amount in the absence of endogenous talin (Fig. 2.2D, N). These results suggest that introducing mutations that impinge on integrin binding in either IBS domain confer only a small reduction in talin recruitment downstream of integrin, while mutating both IBS domains confers a larger reduction on talin recruitment.

In addition to the MTJs, we studied the localisation of GFP-tagged full-length talin rescue constructs in ectodermal tissues that require talin for normal morphogenesis. Wildtype talin-GFP localised to the cell cortices of ectodermal epithelia in stage 12 embryos undergoing germband retraction, and also to the leading edge of the epidermis in stage 15 embryos undergoing dorsal closure (Fig. 2.5). Introduction of mutations into either IBS-1 or IBS-2 in the GFP-tagged full-length rescue constructs did not significantly affect basal cortical enrichment in either stage 12 ectodermal epithelia or stage 15 leading edge cells (Fig. 2.5). However, consistent with our MTJ studies, the double IBS mutant was not enriched at cell cortices in either cell type (Fig. 2.5).

2.2.2 The IBS domains of talin are recruited to integrin by different mechanisms.

Previous studies exploring how the IBS-1 domain of talin interacts with integrins have implicated the distal half of the integrin cytoplasmic tail including the first of its two NPxY motifs [100, 235, 251, 252]. There are conflicting reports as to whether talin IBS-2 also requires the distal part of the integrin cytoplasmic tail [114, 118]. To elucidate the mechanism that recruits the IBS-2 domain of talin to integrin we used an allele of βPS integrin that lacks the second half of the integrin cytoplasmic tail (Fig. 2.1B; Fig. 2.3H-P). This allele, mys<sup>GI</sup>, carries a mutation in a splice acceptor site resulting in truncation of the cytoplasmic tail but does not affect protein stability or surface expression [253]. The GFP-tagged IBS-2 domain
concentrated at MTJs to the same extent in muscles in \( mys^{G1} \) mutant embryos as in wildtype (Fig. 2.3H, J). In comparison, GFP-tagged IBS-1 domain failed to concentrate at MTJs in \( mys^{G1} \) mutant embryos (Fig. 2.3I, K).

We previously showed that full-length talin is recruited by integrin lacking the distal half of the integrin cytoplasmic tail [100]. We confirmed this observation using GFP-tagged full-length talin, which concentrated at MTJs in \( mys^{G1} \) mutant embryos (Fig. 2.3L,M). The GFP-tagged full-length IBS-1 R367A mutant was enriched at MTJs (Fig. 3L, N). However, the GFP-tagged full-length IBS-2 KS>DD and LI>AA talin mutants were not enriched at MTJs (Fig. 2.3L, O, P). This suggests that integrin-mediated recruitment of talin is mediated by interactions of IBS-1 with distal regions and IBS-2 with proximal regions of the integrin cytoplasmic tail. Moreover, the IBS-2 domain can recruit talin to MTJs independently of IBS-1-mediated interactions with the integrin cytoplasmic tail.

### 2.2.3 Both IBS domains of talin contribute to talin function but have different roles during development.

To assess the functions of its IBS domains, wildtype talin was replaced in developing \( Drosophila \) embryos with rescue transgenes containing mutations in the IBS sequences. The ability of wildtype and mutant talin rescue constructs to compensate for the absence of talin was studied by removing talin [using the dominant female sterile germline clone technique [254]] and replacing it with ubiquitously-expressed rescue transgenes. Three major talin-independent developmental processes were quantitatively assayed: dorsal closure (DC), germband retraction (GBR), and muscle attachment at the MTJs (Fig. 2.6). The wildtype talin-GFP rescue transgene, on which all mutant rescue transgenes were based, rescued all null
mutant phenotypes we assayed (Fig. 2.7). Although the rescue obtained with the wildtype talin transgene was nearly complete, a small percentage (1%-5%) of embryos exhibited defects (Fig. 2.6A, G-I; Fig 2.7A-E). In accordance with our published data, we found that talin IBS-1 mutant R367A substantially rescued both integrin-dependent morphogenetic processes GBR and DC [[100]; Fig. 2.6C, G-H]. However, we observed late muscle detachment in a significant proportion of embryos rescued with the talin R367A transgene (Fig. 2.6C, I). This phenotype is different from the complete muscle detachment and rounding observed in talin null mutant flies (Fig. 2.6B) and we have previously shown that this phenotype can be rescued by expression of an activated integrin mutant [100]. In comparison, neither mutant IBS-2 rescue transgene, talin LI>AA or talin KS>DD, substantially rescued the GBR and DC defects in talin deficient embryos (Fig. 2.6D-E, G-H). However, both mutant IBS-2 transgenes partially rescued MTJ defects (Fig. 2.6D-E, I). Furthermore, the double IBS mutant rescue construct, talin R367A; LI >AA conferred very poor rescue of all talin mutant phenotypes assayed (Fig. 2.6F, G-I). These results show that while the IBS domains of talin act partially redundantly during development, they have specific roles during GBR, DC, and muscle attachment.

2.2.4 IBS-2 is required to maintain integrin linkage to talin and other IAC components.

As the proposed function of talin’s IBS domains is to link integrin and talin, we analysed whether talin co-localised with integrin in confocal z-stacks through the embryonic musculature in talin-deficient embryos expressing talin IBS mutant transgenes. In the absence of maternal and zygotic talin expression, talin was not detected at MTJs (Fig. 2.8B). Although muscle tissue architecture was severely disrupted as a result of loss of talin function, integrin
localised to MTJs at levels that are comparable to those observed in wildtype MTJs [Fig. 2.8B; [200]]. When a wildtype talin rescue transgene was introduced into talin-deficient embryos, talin and integrin overlapped at MTJs (Fig. 2.8A, P). In talin-deficient embryos rescued with the IBS-1 mutant transgene talin R367A, talin and integrin also appeared to overlap at MTJs (Fig 2.3C). However, analysis of co-localisation by the Pearson correlation coefficient showed a slight decrease of 12.5% in co-localisation compared to the wildtype rescue transgene, likely due to a slight reduction in talin levels following muscle detachment (Fig. 2.8P). Strikingly, in talin-deficient embryos rescued with the IBS-2 mutant transgenes, talin LI>AA or talin KS>DD, we observed separation between talin and integrin (Figure 2.8D, E; arrows in D’, E’). The Pearson correlation coefficient showed substantial reductions in co-localisation -38% and 32% for LI>AA and KS>DD respectively - compared to the wildtype rescue transgene (Fig. 2.8P). Importantly, even though MTJ architecture was disrupted in talin-deficient flies rescued with either the IBS-1 mutant or with the two IBS-2 mutations, the Pearson correlation coefficients were statistically significantly lower in the IBS-2 mutants (p=0.0009). This data suggests that the IBS-2 domain is essential for maintaining the link between talin and integrin.

Since talin forms a crucial bridge between integrins and the IAC at MTJs [100], we sought to determine whether other IAC components remained associated with integrin when the talin IBS domains were mutated. The distribution of the IAC marker paxillin [239] was used to visualize the IAC in talin-deficient embryos rescued with talin IBS domain mutant transgenes (Fig. 2.8F-O). In the absence of maternal and zygotic talin, talin protein was absent and paxillin was severely reduced at the MTJ, though integrin localisation was comparable to that observed in wildtype MTJs (Fig. 2.8G, L). In comparison, when a wildtype talin rescue transgene was introduced into embryos lacking endogenous talin, paxillin, integrin and talin
co-localised at MTJs (Fig. 2.8F, K). In talin-deficient embryos rescued with the IBS-1 mutant transgene, talin R367A, paxillin localisation largely overlapped with both integrin and talin even when MTJs were disrupted (Fig. 2.8H, M). Moreover, analysis of co-localisation by Pearson correlation coefficient showed that there was no statistically significant difference in co-localisation between paxillin/talin and paxillin/integrin at MTJs in the IBS-1 mutant compared to the wildtype rescue transgene (Fig. 2.8Q, R). In comparison, clear separation was observed between paxillin and integrin at MTJs in talin-deficient embryos rescued with the IBS-2 mutant transgenes talin LI>AA or talin KS>DD (Fig. 2.8I, J; arrows in I’ and J’), but paxillin still overlapped with talin (Fig. 2.8N, O). Analysis of co-localisation using the Pearson correlation coefficient showed that there was a statistically significant reduction in co-localisation in MTJs between paxillin and integrin in the IBS-2 mutants compared to the wildtype rescue transgene (47% and 38% for KS>DD and LI>AA, respectively; Fig. 2.8Q). In comparison co-localisation of paxillin and talin in the IBS-2 mutants was similar to that seen in wildtype (Fig. 2.8R). To further confirm these results, distribution of the protein PINCH [238], an additional IAC component, was examined at MTJs. As was the case for paxillin, when IBS-2 was mutated, PINCH showed reduced co-localisation and separated from integrin (Fig. 2.9D,E), but overlapped and co-localised with talin at MTJs (data not shown). While we observed considerable disruption of MTJ morphology in talin-deficient embryos rescued with the mutant IBS-1 rescue transgene, PINCH and paxillin remained overlapped and co-localised with integrin. We tested the additional IAC markers phospho-FAK [pFAK; [240]] and phospho-tyrosine [pY;[239]] and observed identical results to those shown for PINCH and paxillin for both IBS-1 and IBS-2 mutant transgenes (Fig 2.10). Altogether, these results show
that the IBS-2 domain of talin is not only required to maintain linkage of talin to integrin, but also to maintain association between integrins and the rest of the IAC.

2.2.5 IBS-1 but not IBS-2 is required to maintain integrin linkage to the ECM.

Tiggrin, the RGD motif-containing ECM ligand for αPS2βPS integrins, accumulates at MTJs in the space between adjacent muscles and tendon cells and co-localises with integrin [231, 255]. In talin-deficient embryos rescued with the wildtype talin transgene, tiggrin distribution completely overlapped with integrin (Fig. 2.11A). Furthermore, similar to what we have previously shown, we found that in talin-deficient embryos rescued with the IBS-1 mutant transgene, talin R367A, integrin showed substantial separation from the ECM [Fig. 2.11B, arrows in 11B”; [100]]. However, in talin-deficient embryos rescued with the IBS-2 mutant transgenes, talin LI>AA or talin KS>DD, integrins and the ECM remained co-localised at MTJs (Fig. 2.11C, D). These results indicate that the IBS-1 domain but not the IBS-2 domain of talin is essential for maintaining association between integrins and the ECM.

2.2.6 Mutations in integrin that disrupt binding to talin phenocopy mutations in the talin IBS domains.

To further examine whether the talin IBS domains have different roles in integrin-mediated adhesion, mutations designed to disrupt IBS binding were introduced into the βPS-integrin cytoplasmic tail. The ability of wildtype and mutant rescue constructs to compensate for the absence of βPS-integrin was studied by removing endogenous βPS-integrin [using the dominant female sterile germline clone technique [254]] and replacing it with ubiquitously-expressed wildtype and mutant YFP-tagged βPS-integrins. We ensured that mutant βPS-YFP
transgenes were expressed at similar levels to the wildtype transgene (Fig. 2.4B,C). The wildtype βPS-YFP transgene rescued the embryonic defects associated with loss of integrin function and recruited both talin and the IAC component paxillin at levels similar to those observed in wildtype MTJs (Fig. 2.12A-B, E-I; Fig. 2.13A,D-E; Fig. 2.14). Previous work identified the membrane proximal NPxY motif in the integrin cytoplasmic tail as essential for interaction with the talin IBS-1 domain in vertebrates and flies [69, 100, 251]. We generated a βPS-integrin transgene containing the point mutation N828>A in the proximal NPxY (equivalent to N785A in β1-integrin and N744A in β3-integrin; Fig. 2.1B) and found that it failed to rescue the GBR and DC defects of integrin-deficient embryos, but partially alleviated MTJ defects (Fig. 2.12C, E-G). Moreover, the MTJ defects were similar to those observed in talin IBS-1 domain mutants, as the ECM separated from integrin and analysis of Pearson correlation coefficients showed that there was a statistically significant reduction in co-localisation between integrin and integrin by about 30% in the N828A mutant MTJs compared to those of embryos rescued with the wildtype βPS transgene (Fig. 2.12J, M; arrowhead in J”’). There was some variability observed in this hypomorphic phenotype and some MTJs appeared more disrupted than others (compare Fig 2.13B to 2.12J). Additionally, the N828A mutant βPS-integrin was able to recruit, albeit at reduced levels, both talin and paxillin to MTJs and there was no statistically significant change in co-localisation between integrin and talin, integrin and paxillin, or talin and paxillin (Fig. 2.12H,J,L; Fig. 2.13B,D-E; Fig. 2.14).

Mutations have been identified in the integrin cytoplasmic tail that interfere with its binding to the IBS-2 domain of talin (Rodius et al., 2008). We generated a βPS-integrin rescue transgene containing point mutations in the corresponding residues in fly talin, E810>Q/E817>Q (equivalent to E726>Q/E733>Q in vertebrate β3-integrin; Fig. 2.1B). This
βPS-integrin EE>QQ transgene failed to rescue the GBR and DC defects of integrin-deficient embryos (Fig. 2.12D-F), but partially rescued MTJ defects (Fig. 2.12G). Importantly, the MTJ defects identified were very similar to those observed with the talin IBS-2 mutant rescue transgenes (Compare Fig. 2.12K to Fig. 2.8D, E). While ECM attachment to βPS-integrin EE>QQ was maintained (Fig. 2.12K, M), both talin and paxillin were recruited to MTJs at reduced levels compared to wildtype (see Fig 2.12H,L; Fig 2.13C,D; Fig. 2.14). Although paxillin and talin remained co-localised with one another (Fig. 2.13E), they separated away from the integrin, similar to the IBS-2 mutant rescued talin-deficient embryos. Analysis of βPS EE>QQ mutant embryos by Pearson correlation coefficients revealed approximately 20% reduction in co-localisation between talin and integrin, and a loss in co-localisation of nearly 60% between paxillin and integrin compared to MTJs in embryos rescued with the wildtype βPS transgene (Fig. 2.12K, M; Fig. 2.13C-D). This data shows that in general, the N828A and EE>QQ mutations in the integrin cytoplasmic tail give rise to defects that share similarities with phenotypes caused by mutations in talin that disrupt the functional interaction between integrin and talin.

2.2.7 IBS-1 and IBS-2 confer different, opposing roles in adhesion dynamics at the MTJ.

We wanted to test the hypothesis that different interactions between talin and integrin through IBS-1 or IBS-2 may comprise a mechanism by which the overall stability of integrin-mediated adhesions can be regulated. We have recently shown that the turnover of the integrin adhesion complex can be studied in vivo in live Drosophila embryos using Fluorescence Recovery After Photobleaching (FRAP) to determine the mobile fraction of IAC components [[243]; see Materials and Methods]. We performed FRAP experiments on GFP-tagged mutant
talin rescue and measured their turnover rates at MTJs by determining the mobile fraction (Fig. 2.15). We found that mutating IBS-1 or IBS-2 conferred opposite effects on the mobile fraction of talin: the GFP-tagged IBS-2 mutant, talin KS>DD, had a mobile fraction that was 43% lower than wildtype talin-GFP (Fig. 2.15). Conversely, the IBS-1 mutant, talin R367A, was more dynamic at MTJs, with a mobile fraction approximately 25% higher than wild-type talin-GFP (Fig. 2.15). These results suggest that mutating IBS-1 causes talin to undergo higher turnover while mutating IBS-2 leads to lower talin turnover.

2.3 Discussion

The work presented here is the first comprehensive in vivo analysis of both integrin-binding sites of talin. Studies of the IBS domains of talin are confounded in vertebrates because there are two talin genes that can act redundantly [156]. However, as the fly genome encodes a single talin gene, we were able to overcome this problem. Our results demonstrate that the C-terminal IBS-2 domain of talin is crucial for its function, but that IBS-1 and IBS-2 share some overlapping functions. Specifically, either IBS domain was sufficient to recruit talin to sites of integrin-mediated adhesion. Further, talin mutants bearing mutations in either domain rescued different subsets of talin-dependent processes. Comparatively, when both IBS domains were disrupted, the resulting phenotype was similar to that observed in a talin null fly. Detailed analyses of IBS function during various developmental processes revealed that each IBS is specifically required for a discrete subset of talin functions: IBS-1 had a more pronounced role in linking integrins to the ECM, while IBS-2 was more important for linking
integrins to the intracellular IAC. These results provide important insight into how integrin function is mediated during different developmental processes.

This study utilizes previously identified mutations in talin to disrupt the integrin-binding capacity of talin’s IBS domains. These experiments rely on two assumptions: firstly, that the mutations do in fact abrogate integrin binding, and secondly, that the mutations do not disrupt other protein-protein interaction domains within talin. Several lines of evidence from our previously published results and the work presented here strongly support these assertions. Firstly, when the mutations we used were introduced in the context of the isolated IBS domains (UAS-IBS1-GFP and UAS-GFP-IBS2), they blocked recruitment to integrins at MTJs. Secondly, full length talin transgenes carrying these mutations were less efficiently recruited to MTJs in a wildtype background compared to wildtype transgenes, indicating reduced ability to compete with endogenous talin for integrin binding sites. Further evidence can be derived from experiments using a truncated βPS-integrin, mys$^G1$, which contains only the putative IBS-2 binding site and eliminates the IBS-1 binding site in the distal part of the β-integrin tail. This mutant integrin protein recruited wildtype talin or the IBS-1 mutant talin R367A, but not talin containing mutations in the IBS-2 domain suggesting specific disruption of IBS-1 dependent talin association with integrin. Altogether, our in vivo analyses of R367A, KS>DD, and LI>AA are in line with published biochemical studies that described the ability of these mutations to specifically abrogate integrin binding. Our observations also suggest that the mutations we used did not interfere with other talin functions. For example, we incorporated the GFP reporter at the C-terminal end of the talin molecule and observed normal GFP fluorescence with all IBS domain mutations, indicating proper protein folding downstream of the mutation. Furthermore, full-length talin mutants containing single IBS
mutations localised well in the absence of endogenous talin and retained functionality, as illustrated by their ability to recruit IAC components and to partially rescue muscle attachment at MTJs. In addition, two different sets of mutations in the IBS-2 domain, including those that affect two surface residues (K2094/S2098; [117, 250], gave rise to similar phenotypes. Nonetheless, even though we found no evidence to suggest this, we cannot completely discount the possibility that the mutations we utilized may affect other functions of talin other than integrin binding.

Although a full-length talin mutant containing a double IBS mutation (talin R367A; LI>AA) was not enriched at MTJs in a wildtype background, in a talin null background a small amount of the double IBS mutant was unexpectedly detected at MTJs. This observation can be explained by recent biochemical studies that have shown that the N-terminal head domain of talin interacts with negatively charged lipids in the plasma membrane and may therefore be weakly stabilized in the cell membrane independently of the IBS domains [64, 105, 256, 257]. We did not confirm the phenotypes we observed with the double IBS mutant construct using a second double IBS mutant construct that contained the alternate set of mutations to disrupt IBS-2 (ie. talin R367A; KS>DD rather than talin R367A; LI>AA). However, we find it unlikely analysis of this additional double IBS-2 construct would have given rise to any interesting, significant differences because the phenotypes conferred by the single IBS-2 mutants talin KS>DD mutation and talin LI>AA were so similar to one another.

Interestingly, the phenotypes observed when the NPxY motif of βPS-integrin, which binds to the talin IBS-1 domain, was disrupted were stronger than those seen with the mutant talin IBS-1 rescue transgene. This can explained by previous studies showing that mutations in the NPxY motif can also disrupt the conformation of the other regions of the integrin
cytoplasmic tail including those that interact with the IBS-2 domain (Rodius et al., 2008). Indeed, the overall phenotype of N828A mutation fits somewhere in the spectrum between the phenotypes observed with the IBS-1 and the IBS-2 mutant talin transgenes and thus may represent the combined effects of a severe disruption of binding to the IBS-1 domain with a partial disruption of binding to the IBS-2 domain. Nevertheless, the characteristic defect seen in MTJs in embryos recued with the N828A integrin mutant was highly specific, detachment from the ECM and not from the IAC, a defect that is unique to flies in which the IBS-1 domain of talin has been mutated.

The functional redundancy of the IBS-1 and IBS-2 domains of talin was principally manifested in two ways: first, when either one of the IBS domains was disrupted, talin was still recruited to integrins; second, with only one functionally active IBS, talin recruited downstream components of the IAC. A third manifestation of this redundancy was that the knockout of either IBS domain gave rise to weaker phenotypes at MTJs than knockout of both domains. The principal implication of these observations is that either IBS is capable of mediating the function of talin as an adapter protein, enabling talin to bind integrins and to provide linkage to the cytoskeleton. Another potential implication of this finding is that on many occasions, the precise mechanism by which integrins recruit talin is not important as long as recruitment occurs. This leads to the question of what mechanism determines whether IBS-1 or IBS-2 binds to integrin? Our data shows that IBS-1 and IBS-2 are recruited by separate mechanisms involving independent interactions with different parts of the integrin cytodomain. For example, the IBS-2 but not the IBS-1 domain mediates talin recruitment in the absence of the entire distal part of the integrin cytoplasmic tail. It is therefore likely that the choice between the two IBS domains of talin is regulated by events that occur at the
integrin cytoplasmic tail, perhaps linked to changes in the conformation or activation states of both talin and integrin.

The fact that talin has two different domains that carry out a seemingly identical function, binding to the integrin cytoplasmic tail, is somewhat curious. Previous reports have shown that the IBS-1 domain of talin is essential [68, 70, 100], and our finding that the IBS-2 domain is also required for talin function helps explain why both binding sites have been maintained over hundreds of millions of years of evolution. The essential role of the IBS-1 domain of talin is to regulate inside-out activation of integrins; however, talin has another vital function in integrin-mediated adhesion, which is to link integrins to the IAC. Our data indicate that this function appears to be heavily dependent on the IBS-2 domain. Importantly, ablation of IBS-2 does not prevent the initial recruitment of IAC components, but rather disrupts the attachment between integrins and the assembled IAC. These observations are also supported by a recent study that used FRET-FLIM analysis in cultured cells to demonstrate that IBS-2, but not IBS-1, forms a stable complex with integrin [120]. This corroborates our finding that IBS-2 is required to stably link talin to integrins because although IBS-1 efficiently localises talin to adhesion sites, the interaction between IBS-1 and integrin was not sufficient to stabilize talin in the complex.

It is striking that dynamic morphogenetic processes rely so heavily on the IBS-2 but not on the IBS-1 domain of talin. During dynamic adhesive processes, adhesion complexes must be rapidly assembled and disassembled. For such processes it is likely that the conversion of transient integrin-mediated Cell-ECM adhesion into strong and stable Cell-ECM adhesion, which is the process regulated by IBS-1 [[100]; this study], is not a crucial factor. Furthermore, our FRAP studies suggest that the binding of integrin to talin through IBS-1 or
IBS-2 affects talin turnover in opposite ways: turnover is higher with the IBS-1 mutation and lower with the IBS-2 mutation. We hypothesize that this occurs because the reduced integrin activation that results from mutating IBS-1 leads to a less stable, more dynamic adhesion complex. Conversely, when IBS-2 is mutated, talin can only link to integrin through the IBS-1 domain, leading to increased activation compared to the wildtype. We speculate that increased integrin activation acts to stabilize the integrin adhesion complex. During morphogenesis, it is likely that dynamic adhesion plays a larger role and that as a result, IBS-2 plays a greater role in linking talin to integrin. We propose a model whereby when the talin IBS-2 domain is mutated, talin links to integrin exclusively through IBS-1 which impinges upon the ability of the adhesion complex to be rapidly turned over and leads to defects in morphogenesis.

Another potential reason for the importance of IBS-2 during morphogenesis is that the cytoskeletal and cellular rearrangements that occur during dynamic morphogenetic processes likely require strong linkage between integrins and the IAC. Our data showing separation between IAC markers and integrin at MTJs upon disruption of the IBS-2 domain suggests that IBS-2 maintains the integrin-IAC connection. It is unclear why IBS-2 is particularly important to maintain the integrin-IAC link. One possibility is that the interaction between integrins and the talin IBS-2 domain frees IBS-1 from integrin binding so that the talin head can associate with other cytoplasmic components. The talin head domain could play a vital role in IAC assembly as it contains a multitude of protein binding domains that interact with a variety of IAC components, including Wech, FAK, Ha-RAS, PIP-kinase and others (reviewed in [67][51]). Consistent with this assertion, deletion of the talin head domain severely disrupts talin function (see Chapter 4).
Based on our results, we propose that differential binding by each IBS domain of talin to the integrin cytoplasmic tail comprises an important regulatory mechanism in integrin-mediated adhesion. The ability of integrin to differentially interact with two distinct sites in talin may comprise a regulatory switch between two modes of integrin-mediated adhesion (Fig. 2.16). Our study suggests that IBS-1 function supports stable linkage of integrins to the ECM. In comparison, association of talin with integrin through IBS-2 stabilized connections to the intracellular IAC. It is likely that under certain circumstances either IBS domain is equally capable of mediating the role of talin in integrin mediated-adhesion. However, during crucial periods in animal development, specific functions may be preferentially mediated through integrin binding to either IBS-1 or IBS-2 and it is only then that the choice between the IBS domains becomes important. This differential interaction illustrates one mechanism for how diverse adhesion complex functions can be achieved over the life of an organism.

### 2.4 Materials and Methods

#### 2.4.1 Molecular biology.

The making of *UAS-IBSI-GFP* and *UAS-GFP-IB1-R367A* is described previously [100]. To make *UAS-GFP-IBS2*, sequence corresponding to amino acids 1981-2113 was PCR-amplified from genomic talin and cloned using *PstI* and *SacII* into the *pUAS-mGFP6* plasmid [236]. To amplify *IBS-2* (a.a. 1981-2113) from the genomic *rhea* sequence we used the primer pairs 5’-*tatactgcag tcgcgtggaactcaggcgtg*-3’ and 5’-*tataccgcggtcacttgccggaggccaacttag*-3’. The LI>AA and KS>DD mutant IBS2 domains were made using QuikChange Lightning
Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and the following mutagenesis primers (5’ shown only, changes indicated in bold): LI>AA: 
\[
gtagcctctgcacttggtgat/gcg/gcgt/aactgcactaagttggcctcc
\]
and KS>DD: gtcataatgcgctgat/gac/gatggctgatcggtaaatgggctcc. Full length talin-GFP rescue constructs were all based from the \(p\text{Ubi-Talin}[\text{EGFP}]\) construct [243] except talin R367A which was adapted from \(p\text{Ubi::Talin}(R367A)\) [100] to include a GFP as outlined in [243]. The IBS double mutant talin R367A; LI>AA transgene was constructed by sub-cloning a 2207bp section with KpnI and StuI from the \(p\text{Ubi-talin}(R367A)\) construct into the talin LI>AA transgene. \(p\text{Ubi-\beta PS-YFP}\) was made as described in [243]. Mutations were introduced using site-directed mutagenesi and the following mutagenesis primers: [N828A] 5’-tgggatacgggcgag/gct/cccatctacaagcag-3’; [E810>Q] 5’- cacgatggcgag/gct/cccatctacaacgag-3’; [E817>Q] 5’-cggcttcgagaag/gct/cccatctacaagcag-3’. All transgenics were generated by Bestgene (Chino Hills, Ca).

### 2.4.2 Fly stocks and genetcs.

In order to obtain embryos maternally and zygotically null for talin or \(\beta\)PS, germ line clones (GLCs) were generated according to the dominant female sterile technique (Chou and Perrimon, 1996). For \(rhea\) GLCs, males of the genotype \(y,w,hs\text{F}\text{lpl/Y;\text{Ovo}}^{D1},\text{FRT}^{2A}/\text{TM3}\) were crossed to \(rhea^{79},\text{FRT2A}\) females carrying each of the different talin rescue transgenes so the mutant protein was supplied maternally and zygotically. Progeny of this cross was heat shocked in larval stages for 2 hours at 37°C for 3 consecutive days, and \(\text{transgene;}rhea^{79},\text{FRT2A/Ovo}^{D1},\text{FRT2A}\) females were crossed to \(rhea^{79}\) males. Embryonic progeny were collected for fixation every 24 hours.
For *mys* GLCs the same procedure were followed using the lines *Ovo*<sup>D1</sup>*FRT*<sup>101</sup>*/Y; hsFlp<sup>38</sup> and *mys*<sup>XG43</sup>*FRT*<sup>101</sup>*/FM7, *Kr::GFP*. Flies of the genotype *Ovo*<sup>D1</sup>*FRT*<sup>101</sup>*/mys*<sup>XG43</sup>*FRT*101 were selected for and then crossed to males carrying each of the βPS rescue transgenes of interest.

For analysis of talin localisation in *mys*<sup>G1</sup> background, *y,w,mys*<sup>G1</sup>*FRT*18A/*FM7,Kr::GFP* females were crossed to males bearing each talin transgene and embryos lacking the *Kr::GFP* balancer were selected for analysis.

### 2.4.3 Immunohistochemistry, confocal microscopy, and image analysis.

Embryos were heat-fixed (Tanentzapf and Brown, 2006) for most experiments with the exception of phalloidin stainings which required a methanol-free protocol whereby embryos were fixed for 20 minutes in 4% paraformaldehyde, devitellinated in 2:1 heptane and 90% ethanol, and permeabilized in 0.2% Triton X-100.

Antibodies used were as follows: α-MHC, 1:200 (Dan Kiehart, Duke University, Durham, NC), rabbit α-PINCH (1:1000; Mary Beckerle, Huntsman Cancer Institute, UT), rabbit α-talin (E4; 1:500), mouse α-talin (E17B,1:50), rat α–αPS2 (7A10, 1:100), α–βPS (Nick Brown, Gurdon Institute, Cambridge, UK), rabbit α-Paxillin (1:1000; [239]), mouse α-Tiggrin 1:500 (Liselotte Fessler, UCLA, CA), rabbit α–GFP (A6455, 1:1000; Invitrogen), Rhodamine-conjugated phalloidin (Invitrogen) was used to stain actin filaments (1:400). Fluorescently-conjugated Alexa 488, Cy3 and Cy5 secondary antibodies were used at 1:400 dilution (Molecular Probes).
Images were collected using an Olympus FV1000 confocal microscope with a UplanSApo 60/1.35 oil objective and a UplanFL N 40x/1.30 oil objective and processed using Adobe Photoshop. All quantification of fluorescence at MTJs was done using ImageJ (NIH, Bethesda, MD). Statistically significant differences in localisation between wildtype and mutant constructs was assessed by the two-tailed t-test in all cases, except when we sought to compare between multiple constructs, where one-way ANOVA was used. Statistical analysis was carried out using Prism4 software (GraphPad, La Jolla, CA). For intensity traces across MTJs, the ImageJ plot profile tool was used to determine the average signal intensity across the boxed area indicated on images. Each channel was independently normalized from unprocessed, gray scale images so the peak intensity of each channel across the area of interest was set as 100% and the lowest intensity was set to be 0%. In the cases where one of the two signals was mislocalised (as in Fig. 11B’’’ for example), 100% was set as the maximum intensity of the localised channel for both channels.

To determine co-localisation, we used the co-localisation feature in Olympus FluoView1000 software to determine the Pearson Correlation Coefficient. A region of interest was drawn around single 1.0µm section images in the middle plane of each MTJ. Images were taken so that the signal of each marker being measured was set to just below saturation at the MTJ prior to analysis. At least three MTJs in three different embryos were selected for each set of measurements.

2.4.4 Western blot analysis.

For the western blot data shown in Figure 2.4, protein was isolated from whole flies and blotted according to standard procedures using primary antibodies to GFP (A6455,
1:1500, Invitrogen) and actin (8224, 1:5000, Abcam) and secondary antibodies conjugated to InfraRed (IR) dyes, IR680 and IR800 (LiCor Odyssey, 1:15,000). Blots were scanned on a LiCor Odyssey Imaging System.

2.4.5 qPCR.

For the qPCR data shown in Figure 4 total RNA was isolated from whole flies using TRIzol (Invitrogen) and treated with DNase (Fermentas). 1000 mg of total RNA was converted to cDNA using the qScript™ cDNA Synthesis Kit (Quanta Biosciences). Subsequently, qPCR was performed using the PerfeCTa™ SYBR® Green FastMIX™ ROX kit (Quanta Biosciences). As all βPS transgenes were YFP-tagged, we could to quantify transgenic mRNA transcript levels by assaying YFP expression using primer pairs 5’-ggcacaagctggagtacaac-3’ and 5’-agctcaggtagtggttgtc-3’. GAPDH mRNA levels were assayed as an internal control using the primer pairs 5’-aaagccggcagtctgtagc-3’ and 5’-gacatcgatgaaggatcg-3’. Expression changes were determined by using the comparative Ct method for relative quantitation.

2.4.6 FRAP.

Stage 17 embryos were collected and prepared for FRAP as in Yuan et al (2010). Briefly: Embryos heterozygous for each transgene were collected from apple juice plates, dechorinated in 50% bleach for 3 minutes, washed with PBS, and mounted on glass slides in PBS. FRAP analysis was carried out at room temperature. Photo-bleaching was performed using a 405 nm laser at 30% power with the Tornado scanning tool (Olympus) for 2 s at 100 msec/pixel. Fluorescence recovery was recorded over 5 min at 1 frame every 4 s. To control
muscle twitching in and out of focus, multiple regions of interest (ROI) were selected in non-photobleached regions; only samples for which intensities within control ROIs remained steady throughout the FRAP experiment were used. Recovery data were further analyzed using Prism software (GraphPad, La Jolla, CA): mobile fraction was calculated as previously described [258]. Statistical tests (t-test, analysis of variance [ANOVA] test) were carried out using Prism.
Figure 2.1. Talin IBS-2 and the β-integrin cytoplasmic tail are conserved between *Drosophila* and humans.

(A) Alignment of fly talin and human talin IBS-2. Black indicates identical residues and grey indicates similar residues; red-boxed residues were mutated in this study as marked. (B) Alignment of the cytoplasmic tails of human β3-integrin and fly βPS integrin. Black indicates identical residues and grey indicates similar residues. Red-boxed residues mutated in this study; arrowhead marks position of the *mys*<sup>Gr</sup> lesion.
Figure 2.2. *Drosophila* talin contains two conserved integrin binding sites that are each sufficient to localise talin to integrins at MTJs.

(A) Schematic of talin constructs used in this study. Integrin binding domains are shown in green. (B) Relative subcellular localisation of GFP-tagged IBS domains to MTJs compared to cytoplasm when expressed in the embryonic musculature using the dMef2-GAL4 driver. (C, D) Relative localisation of ubiquitously-expressed full length talin-GFP transgenes to MTJs compared to cytoplasmic fluorescence in WT (A) and talin-deficient stage 17 embryos (B). Recruitment to MTJs was quantified by determining the average ratio of GFP fluorescence (B, C) or anti-talin antibody signal (D) at MTJs to cytoplasmic fluorescence for at least 3 MTJs in at least 5 embryos. Values >1 indicate enrichment at MTJs. (Error bars represent the s.e.m. ***p ≤ 0.0001, **p<0.001, and *p<0.05 compared to the recruitment of each WT IBS domain (B) or full length talin-GFP in WT (C) or talin-deficient (D) backgrounds.) (E-N) Representative confocal images of muscles expressing full length talin-GFP constructs in WT (E, G, I, K, M) and talin-deficient (F,H,J,L,N) stage 17 embryos. (E-F) WT full-length talin-GFP; (G-H) IBS-1 mutant talin (talin R367A); (I-L) IBS-2 mutant talin (I-J, talin KS>DD; K-L, talin LI>AA); (M-N) double IBS mutant talin (talin R367A, LI>AA); arrows indicate MTJs. (n.s = not significant; a.u. = arbitrary units.) Scale bar = 20 µm.
Figure 2.3. Either IBS domain of talin is sufficient to localize talin to integrins at MTJs by independent mechanisms that depend on different parts of the β-integrin cytodomain. (A-G) Single confocal sections of images of GFP-tagged IBS domains expressed in the embryonic musculature under the control of the MEF2-GAL4 tissue driver. Both IBS-2 (A) and IBS-1 (B) localised to MTJs (arrows) in wildtype embryos but failed to localise in integrin mutant backgrounds (C-D). Mutations in conserved residues in GFP tagged IBS-1 (F) or IBS-2 (E,G) disrupted localisation to MTJs. (H-K) Localisation of the GFP-tagged talin IBS domains IBS-2-GFP (H,J) and IBS-1-GFP (I,K) in the integrin mutant mysG1 background compared to wildtype background. Although both domains were recruited to MTJs in a wildtype background, in mysG1 mutant embryos GFP-IBS2 but not IBS1-GFP was recruited to MTJs. Quantification of GFP fluorescence at MTJs determined as in Figure 1B. **p<0.0001 for IBS1-GFP in wildtype vs. mysG1 background. (L-P) Localisation of the GFP-tagged full length talin in mysG1 embryos: wildtype talin-GFP (L,M), IBS-1 mutant (talin R367A; L,N), and
IBS-2 mutants (talin LI>AA, O, and talin KS>DD, P). The wildtype and IBS-1 mutant but not IBS-2 mutant talin are recruited in a mys\textsuperscript{G1} background. (Error bars in L indicate s.e.m., **p >0.001 and ***p>0.0001 compared to recruitment of wildtype full-length talin). All images were taken in live stage 17 embryos. Scale bars = 20mm.

![Figure 2.4](image)

**Figure 2.4. Expression levels of transgenic talin and βPS-integrin rescue constructs.** (A,B) Quantification of transgenic protein expression level determined by western blotting for GFP to detect pUbi-talin-EGFP (A) or pUbi-βPS-YFP (B) averaged between two independent experiments. β-actin was used as a loading control. We found no significant difference in expression among talin (A) or βPS (B) transgenes. (C) Quantitative PCR analysis of βPS-YFP transgenes averaged between two independent experiments performed in triplicate (see Supplemental Experimental Procedures) and normalized to GAPDH expression. We found no significant difference in transcript level between wild type and mutant transgenes. Error bars represent S.E.M. (A, B) or S.D. (C).
Figure 2.5. Localization of fluorescently-tagged full length talin protein in the ectoderm and leading edge epidermis.

(A-D, F-I) Single confocal sections of live embryos expressing full length talin transgenes: wild-type talin-GFP (A, F), IBS-2 mutant talinEGFP*KS>DD (B, G), IBS-1 mutant talinGFP*R367A (C, H), and double IBS mutant talinEGFP*R367A; LI>AA (D, I). (A-E) The ectoderm of stage 12 embryos undergoing germband retraction is shown localization was quantified (E) by measuring the enrichment of fluorescence at the lateral cortex compared to the cytoplasm. A ratio of <1 indicates failure to localize to the cortex. At least 5 cells were measured in at least 5 different embryos. (F-J) The leading edge epidermis of stage 15 embryos undergoing dorsal closure is shown and localization is quantified (J) by measuring the ratio of signal at the leading edge to the cytoplasmic signal of epidermal cells adjacent to the leading edge. A ratio <1 indicates failure to localize to the leading edge. At least 5 cells were measured in at least 5 different embryos. ***p<0.0001 for localization of talin R367A; LI>AA compared to wild type talin-GFP indicating the double IBS mutant localised to neither the leading edge epidermis nor the ectodermal cell cortex. Error bars indicate s.e.m. Scale bar = 10 µm.
Figure 2.6. IBS-1 and IBS-2 mediate discrete subsets of talin function in development. (A-F) Confocal z-stacks of whole-mount stage 17 (A) WT rescue embryos, (B) talin-deficient embryos and (C-F) talin-deficient embryos expressing full-length (C) IBS-1, (D,E) IBS-2 or (F) double mutant transgenes (M/Z germline clones, see Materials and Methods). Embryos were labeled for F-actin (red) and integrin (green). Loss of talin led to failure in DC and GBR as well as MTJ defects (B), which were rescued by the WT talin-GFP transgene (A). (G-I) Penetration of DC (G), GBR (H) and MTJ defects (I) in WT, talin-deficient and transgenic talin rescue embryos. ***p<0.0001, **p<0.001, and *p<0.05 compared to the penetration of each phenotype in non-rescued, talin/-/- embryos; phenotypes were scored for at least 30 embryos/genotype. Scale bar: 100 µm.
The pUbiTalin::EGFP transgene fully rescues embryonic defects in talin-deficient embryos. (A,B) Whole mount stage 17 embryos labeled for talin (green), myosin heavy chain (MHC, red) and αPS2 integrin (blue) showing representative phenotypes of wild type (A) and talin-deficient (M/Z germline clones, see Materials and Methods) flies rescued with a wildtype talin transgene (pUbiTalin::EGFP). (C-E) Quantitative phenotypic analysis of the rescue of talin loss-of-function phenotypes by wildtype transgene compared to non-rescued talin mutants and wildtype flies: penetrance of germband retraction defects (C), dorsal closure defects (D), and MTJ defects (E) scored for at least 30 embryos per genotype. (F-K) MTJs of wild type (F, H,
J) and talin mutant flies rescued with the pUbiTalinEGFP transgene (G, I, K). (F, G) Talin (green) is recruited and overlaps with αPS2 integrin; the muscle cytoskeleton is highlighted by MHC (blue). Traces show average intensity profile of αPS2-integrin and talin across 20µm width of boxed area. (H, I) MTJs showing tiggrin (red) was deposited normally and colocalises with αPS2 integrin (green). Traces show average intensity profile of αPS2-integrin and tiggrin across width of boxed area. (J, K) The IAC component paxillin is recruited to MTJs and colocalises with αPS2-integrin and talin. Traces show average intensity profile of αPS2-integrin and tiggrin across width of boxed area. (L-O) Pearson correlation coefficients for assessing colocalisation of talin and αPS2 (L), tiggrin and αPS2 (M), paxillin and aPS2 (N), and paxillin and talin (O) in wild type flies and in talin-null flies rescued with the wildtype talin-GFP transgene. Scale bars (A,B) 100 µm, (F-K) 20 µm.
Figure 2.8. IBS-2 is required to maintain the connection between integrins and the IAC. (A-O) Confocal z-stacks of MTJs in stage 17 talin deficient-embryos rescued with full length talin transgenes. Embryonic muscles were labeled for talin (green, A-E; red, K-O), αPS2-integrin (red, A-J), and paxillin (green, F-O). (A’-O’) Average intensity profiles of (A’-E’) talin and αPS2-integrin, (F’-J’) αPS2 and paxillin, and (K’-O’) paxillin and talin across the widths of the boxed areas indicated in corresponding images. MTJs in WT talin rescue embryos exhibited complete overlap between talin and αPS2 integrin (A), paxillin and αPS2 integrin (F) and paxillin and talin (K). In talin-deficient embryos, integrin (B,G) concentrated...
at the MTJ but paxillin did not (L). IAC components largely co-localised in talin-deficient embryos rescued with IBS-1 mutant talin. By contrast, talin (D,E) and paxillin (I,J) separated from αPS2 integrin (arrowheads, D’-E’,I’-J’) in talin-deficient embryos rescued with IBS-2 mutant talin (talin KS>DD, D,I; talin LI>AA, E,J). However, talin and paxillin mostly co-localised in IBS-2 mutant rescues (N, O). (P-R) Pearson correlation coefficients indicate the extent of co-localisation between talin and αPS2 (P), paxillin and αPS2 (Q), and paxillin and talin (R) in talin-null embryos rescued with the indicated transgenes. (**p<0.001, *p<0.05 compared to the Pearson correlation coefficient for the corresponding markers in the WT talin rescue. Error bars represent s.e.m.) Scale bar = 20 μm.
Figure 2.9. Talin IBS-2 maintains the link between integrins and the IAC component PINCH.

(A-E) Confocal z-stack images of MTJs in stage 17 talin deficient embryos (M/Z germline clones, see Materials and Methods) rescued with talin transgenes. Embryonic muscles were labeled for PINCH (green), αPS2-integrin (red) and Myosin Heavy Chain (to mark muscle cytoskeleton, blue). Traces in A''-E'' show average intensity profile of PINCH and αPS2-integrin across width of the boxed area in A''-E''. (A) Normal MTJs in wildtype talin-rescued embryos exhibit complete overlap between PINCH and αPS2 integrin. (B) In talin-deficient embryos, PINCH staining was absent from MTJs (our PINCH anti-sera non-specifically labels the chordotonal organs; see Clark et al, 2003) but integrin concentrates at the MTJs. (C)
PINCH and αPS2 integrin largely overlap in talin-deficient embryos rescued with an IBS-1 mutant talin (talin R367>A). (D, E) PINCH and αPS2 integrin separated from each other (arrowheads in D''' and E''') in talin-deficient embryos rescued with IBS-2 mutants (talin KS>DD, D; talin LI>AA, E). (F) Pearson correlation coefficients for assessing colocalisation of PINCH and αPS2 integrin in talin-deficient flies rescued with wild type and IBS mutant transgenes. ***p<0.0001 compared to colocalisation of wildtype talin-GFP rescued talin-/- embryos. Scale bar = 20µm.

Figure 2.10. Localisation of IAC components phospho-tyrosine and phospho-FAK in the Drosophila embryonic MTJ.
(A) pY localisation in WT and IBS-2 mutant talin KS>DD rescued talin-/- flies. Although the high background renders these images unsuitable for quantitative analysis, pY noticeably separates from the integrin, along with other IAC components, including talin. (B) pFAK staining gives high background in WT and IBS-2 mutant KS>DD rescued talin-/- flies, and thus, these images are not amenable to further analysis.
Figure 2.11. IBS-1, but not IBS-2, is required to maintain the connection between integrin and ECM ligands. 

(A-D) Confocal z-stacks of MTJs in stage 17 talin-deficient embryos rescued with talin transgenes. Embryonic muscles were labeled for the ECM protein Tiggrin (white, A-D; red, A’-D’) and αPS2 integrin (white, A’-D’; green, A’’-D’’). (A’’’-D’’’) Average intensity profiles for αPS2-integrin and tiggrin across the widths of the boxed areas in A’’-D’’. Tiggrin and αPS2 integrin completely overlapped at MTJs in WT talin rescue embryos (A, A’’’). Tiggrin and αPS2 integrin separated in talin-deficient embryos rescued with an IBS-1 mutant (arrowheads, B’; B’’’). Overlap between tiggrin and αPS2 integrin was maintained in talin-deficient embryos rescued with IBS-2 mutants (C-D, C’’’-D’’’). (E) Pearson correlation coefficients indicate the extent of co-localisation between tiggrin and αPS2 for talin-null flies rescued with the indicated transgenes. *p<0.05 compared to the Pearson correlation coefficient for tiggrin and αPS2 in the WT rescue. Error bars represent s.e.m. Scale bar = 20 μm.
Figure 2.12. Two distinct talin-binding sites in βPS-integrin are essential for fly development.
(A-D) Confocal z-stacks of whole-mount stage 17 (A) WT βPS-YFP rescue embryos, (B) βPS-deficient embryos and (C,D) βPS-integrin-deficient embryos rescued with mutant βPS-integrin transgenes, (C) βPS*N828A or (D) βPS*EE>QQ (M/Z germline clones, see Materials and Methods). Embryos are labeled for βPS-integrin (green) and F-actin (red). Loss of βPS-integrin leads to failure in DC and GBR as well as MTJ defects (B); the WT βPS-YFP transgene rescued these embryonic phenotypes (A). (E-H) Penetration of GBR (E), DC (F), and MTJ (G) defects in embryos of the indicated genotypes. ***p<0.0001, **p<0.001, and *p<0.05 compared to the percent penetrance of each phenotype in non-rescued, βPS/- embryos; n>30 embryos/genotype.) (H) Talin recruitment by βPS rescue constructs, measured as the ratio of talin antibody signal at the MTJ compared to the cytoplasm. A ratio of 1 indicates that talin was not recruited. ***p ≤ 0.0001 compared to talin recruitment by WT βPS-YFP. (I-K, I''-K'') Confocal z-stacks of MTJs in stage 17 βPS-deficient embryos rescued with the indicated transgenes. Embryonic muscles were labeled for talin (green), αPS2-integrin (red) and tiggrin (blue). (I'-K', I'''-K''') Average intensity profiles for talin and αPS2-integrin (I'-K') and tiggrin and αPS2 (I'''-K''') across width of the boxed areas in corresponding images. Talin, αPS2 integrin and tiggrin completely overlapped at MTJs in WT βPS rescue embryos (I-I'''). Talin and αPS2 integrin largely overlapped in βPS-deficient embryos rescued with βPS*N828A (J-J'), however, αPS2 detached from the ECM (J''; arrowhead, J''). Talin and αPS2 integrin separated from each other (K; arrowheads, K') in βPS-deficient embryos rescued with βPS*EE>QQ, but attachment between the ECM and integrins was maintained (K'', K'''). (L,M) Pearson correlation coefficients indicate the extent of co-localisation of αPS2 and talin (L) and tiggrin and αPS2 (M) in βPS-deficient embryos rescued with the indicated transgenes. **p<0.001, and *p<0.05 compared to the Pearson correlation coefficient determined for each of the corresponding markers in the WT βPS rescue. Error bars represent s.e.m. Scale bars: (A-D) 100 µm; (I-K) 20 µm.
Figure 2.13. Mutations in each talin binding site of βPS do not disrupt localization of talin or paxillin but phenocopy talin IBS mutants.

(A-C) Confocal z-stacks of embryonic muscles labeled for paxillin (green), αPS2 integrin (red, A-C) and talin (red, A'′- C'′). (A'′-C', A'′′-C'′′) Average intensity profiles for paxillin and αPS2 integrin (A'′-C') and paxillin and αPS2-integrin (A'′′-C'′′) across the width of the boxed areas in the corresponding images. βPS-deficient embryos rescued with either WT βPS-YFP (A,A′) or βPS*N828A-YFP (B,B′) exhibited overlap between paxillin, αPS2-integrin and talin. In βPS-deficient embryos rescued with βPS*EE>QQ, paxillin separated from αPS2 integrin (C; arrowheads, C′) but co-localised with talin (C''',C''′′). (D,E) Pearson correlation coefficients indicate the extent of co-localisation between paxillin and αPS2 (D) and talin and paxillin (E) in βPS-deficient embryos rescued with the indicated transgenes. ***p<0.0001 compared to the Pearson correlation coefficient determined for each of the corresponding markers WT βPS rescue. Error bars represent s.e.m. Scale bars = 20 μm.
Figure 2.14. Talin recruitment by βPS-integrin point mutants.
(A-D) Confocal z-stack images of stage 17 embryonic MTJs labeled for αPS2 integrin (red), talin (green), and MHC (blue) to show the muscle cytoskeleton. (A) Neither talin nor αPS2-integrin localises to MTJs in βPS-integrin-deficient embryos (m/z germline clones; see Materials and Methods). (B-D) βPS-deficient embryos rescued with a wildtype βPS (B), N828>8 βPS (C), or EE>QQ βPS (D) transgene show colocalisation of integrin and talin at MTJs, (B’-D’) talin recruitment to MTJs is shown in gray scale.
Figure 2.15. Mutations in IBS-1 and IBS-2 confer opposing effects on adhesion dynamics. (A) Fluorescence Recovery After Photobleaching (FRAP) measured at MTJs revealed differential recovery dynamics among talin mutants. (B) The mobile fraction of WT talin-GFP at MTJs was higher than IBS-2 mutant talin KS>DD, but lower than IBS-1 mutant talin. ***p<0.0001 and *p<0.05 compared to the recovery of WT talin-GFP. Error bars represent s.e.m.
Figure 2.16. Proposed model of the functions of talin IBS-1 and IBS-2.
CHAPTER THREE: Rap1-Dependent Control of Talin

Autoinhibition is Required for Morphogenesis

3.1 Introduction

Integrins connect to the cytoskeleton through an intracellular adhesion complex (IAC); changes to the protein composition and interactions within the IAC have important implications for integrin-dependent cellular behaviors [94, 259-261]. Talin is an essential IAC component [67, 200] containing a conserved, integrin-binding FERM domain at its N-terminus, and an actin-binding domain at the C-terminus of its helical rod domain [67]. Structural studies identified residues in both the talin FERM and rod domains that mediate autoinhibition (Figure 3.1A) [108, 130]. The same region of the talin FERM domain that binds integrin also binds the rod to mediate autoinhibition [108]. It has been proposed that talin autoinhibition may provide a mechanism to down-regulate talin-dependent integrin activation and blocking talin autoinhibition leads to integrin activation [108, 130]. The biological role of talin autoinhibition is currently not well defined but initial results in cell culture suggest that it plays an important role as expression of autoinhibition-impaired talin results in increased integrin activation and altered cell spreading [130, 131].

Here, we demonstrate that targeted disruption of talin autoinhibition gives rise to morphogenetic defects during fly development and specifically that dorsal closure (DC), a process that resembles wound healing, is delayed. Impairment of autoinhibition leads to reduced talin turnover at and increased talin and integrin recruitment to sites of integrin-ECM attachment. Finally, we present evidence that talin autoinhibition is regulated by Rap1-
dependent signaling. Based on our data we propose that talin autoinhibition provides a switch for modulating adhesion turnover and adhesion stability that is essential for morphogenesis.

3.2 Results

3.2.1 Talin autoinhibition is a conserved mechanism between vertebrates and Drosophila.

We hypothesized that the mechanism of autoinhibition is conserved between flies and vertebrates. The autoinhibitory regions have been mapped to the F3 lobe of the FERM domain (residues 309-400 in Human Talin1; 318-409 in Drosophila Talin), and a region of the rod called R9, which forms an amphipathic helical bundle (residues 1655-1826 in mammals; 1662-1831 in fly) [108]. The F3 domain is highly conserved across species, with 85.7% protein sequence similarity and 74.7% identity between human Talin1 and fly talin (Figure 3.2A). The protein sequence of R9 is also highly conserved with 56.3% similarity and 33.5% identity (Fig. 3.2B). We used homology modeling to predict the structure of the rod R9 domain based on the NMR structure of mouse talin and found the fly structure closely resembles that of mouse (Fig. 3.1B,C). Notably, four negatively charged surface residues in the rod that are important for autoinhibitory interactions between the FERM and the rod domains are conserved in sequence and arrangement between flies and humans (Fig. 3.1B,C). To quantify differences in secondary structure between the mouse NMR structure and the predicted fly structure, we calculated the root-mean square deviation (RMSD) of the superposition of the two structures (Fig. 3.1D). We obtained a RMSD of 0.148Å for 635 aligned atoms suggesting the two structures are very similar. Homology modeling of the FERM domain also showed excellent conservation between fly and vertebrate (P.L. & F.V.P;
data not shown). We also used NMR spectroscopy to show that the fly R9 domain adopts a stable globular conformation in vitro, similar to the mouse protein homolog (Fig. 3.1E,F).

Altogether, our homology modeling and NMR data suggest that the domains of mammalian talin and fly talin involved in autoinhibition are likely structurally conserved. Importantly, NMR spectroscopy confirmed an interaction between F2-F3 and R9 of fly talin (Fig. 3.1G). This result further confirms the notion that this interaction, which mediates autoinhibition, is conserved between flies and vertebrates.

We sought to design a fly mutant that would specifically disrupt talin autoinhibition. In the R9 domain, we chose to introduce a mutation that was shown, in vitro, to completely abrogate binding with the FERM domain and thus block autoinhibition [108]. This mutation changes a conserved glutamate residue in R9 (E1777 in fly; E1770 in mammalian talin) to an alanine residue (E1777A). NMR analyses demonstrated that the spectra of the region of talin containing the E1777A strongly resembled the spectra of the wild-type region indicating that the mutation does not disrupt protein folding (Fig. 3.1F). It was not feasible to choose a mutation in the FERM domain to abrogate autoinhibition for two reasons. Firstly, there have only been two mutations in the FERM domain that have been described to disrupt autoinhibition: the role of the first, M319A (equivalent to M328 in flies), is the subject of an unresolved dispute [108, 130, 140]. Secondly, the other mutation described to disrupt autoinhibition K324D [108] (equivalent to K333D in flies) is adjacent to a residue that is critical for talin function (L325 in vertebrates; L334 in flies) [73, 74, 131]. Moreover, this region of the FERM domain is packed with interaction sites for talin binding partners (Supplemental Fig. 3.2A). These factors would make it very difficult to interpret, in vivo, the phenotype of mutations in the FERM domain that disrupt talin autoinhibition.
3.2.2 Disrupting talin autoinhibition results in multiple morphogenetic phenotypes including delayed dorsal closure.

To assess the role of talin autoinhibition, wild-type (WT) endogenous talin was replaced in developing Drosophila embryos with rescue transgenes containing the E1777A mutation (see Supplemental Experimental Procedures). Previous analysis has shown that a ubiquitously expressed WT talin rescue transgene (talinGFP) rescues the embryonic lethality that results when embryos lack both maternal and zygotic talin protein ([260]; Fig. 3.3A,B). In comparison TalinGFP*E1777A failed to rescue the lethality associated with loss of talin (Fig. 3.3C,D). The ability of talin transgenes to rescue talin mutants was assayed in the context of three different integrin-dependent processes. Two of these, DC and Germband Retraction (GBR), represent dynamic morphogenetic processes while the third, muscle attachment, represents stable long-term adhesion. While talinGFP fully rescued GBR and DC, TalinGFP*E1777A only gave a partial and inconsistent rescue (Fig. 3.3E,F). DC occurs late in fly embryogenesis and involves the migration of two epidermal sheets over an extra-embryonic epithelium called the amnioserosa (AS); the AS actively contributes to DC [14, 262]. The end result of DC is to create a continuous epidermis on the dorsal side of the embryo. Of embryos rescued with talinGFP*E1777A 27.3% (n=99) failed to complete DC compared with 49.2% (n=53) of talin null embryos and 8% (n=57) of talinGFP rescued embryos (Fig. 3.3E). However, closer examination of earlier stage embryos revealed a more penetrant phenotype (Fig. 3.3G-H); DC normally concludes at stage 15 in talinGFP-rescued embryos (89% completion rate/stage 15; n=9) but this was not the case for the majority of talinGFP*E1777A rescued embryos (22.2% completion rate/stage 15; n=18). Therefore, talin
mutants rescued with talinE1777A exhibited delayed DC (Fig. 3.3H). We confirmed that DC was delayed in talinGFP*E1777A rescued talin mutants using live time-lapse imaging of rescue embryos (Fig. 3.3G, Movies S1-S2).

3.2.3 The talin autoinhibition mutant does not impinge on talin protein expression, stability, or sub-cellular localization.

A possible explanation for the delayed and incomplete DC observed in TalinGFP*E1777A-rescued embryos is insufficient expression of the mutant talin. Quantitative RT-PCR analysis revealed that transcript levels of talinGFP and the talinGFP*E1777A mutant were approximately equivalent (Fig. 3.4A). Western blot analysis showed that talinGFP*E1777A protein levels were slightly less than those of the talinGFP WT transgene (Fig. 3.4B). However, the mutant transgenic protein is still present at levels that are comparable to, and even slightly higher than, the levels of endogenous talin protein since the use of the ubi promoter results in slight over-expression of both talinGFP*E1777A and TalinGFP relative to endogenous protein (Fig 3.4B-C). Intriguingly, we observe a slight difference in size between talinGFP and talinGFP*E1777A, but we have no evidence to suggest that this has any functional consequence. Importantly, we could not detect a reduction in talin levels via antibody staining at myotendinous junctions (MTJs) suggesting that talinGFP*E1777A transgene expresses sufficiently (Fig. 3.5). We also quantified the recruitment of WT talinGFP and talinGFP*E1777A to the prominent integrin adhesions at the MTJs of embryonic muscles using our established protocol [235, 260]. TalinGFP*E1777A was recruited to sites of integrin-mediated adhesion at MTJs better than TalinGFP (Fig. 3.4D-F). This result is reminiscent of recent reports in cultured cells showing that mutating the talin
rod to prevent autoinhibition results in increased talin localization in the membrane fraction [140]. Altogether, the defects we observe in talinGFP*E1777A mutant embryos are likely not caused by reduced expression and/or mislocalization of talin but by the specific effects of the mutation.

3.2.4 Impaired adhesion complex assembly does not underlie the phenotype conferred by the talin autoinhibition mutant.

To investigate whether the TalinGFP*E1777A impairs the assembly of the IAC and/or its attachment to the ECM we analyzed the fly MTJs as they provide an established and quantitative model to study disruptions in IAC recruitment and ECM attachment [100, 235, 243, 260]. We did not find any defects in MTJ integrity, IAC recruitment or ECM attachment in talin mutant embryos rescued with TalinGFP*E1777A (Fig. 3.5). Previous studies suggested that the ability of talin to autoinhibit might comprise a mechanism to modulate vinculin recruitment and actin association. However, we were unable to find any differences in either actin or vinculin recruitment (Fig. 3.5E, Fig3.6A-D). Additionally, vinculin was not expressed in the AS providing further evidence that a disruption in vinculin binding to talin was unlikely to underlie the dorsal closure defects we observed in the talinGFP*E1777A-rescued embryos (Fig. 3.6E). We also used gel filtration to confirm that the R9 of the rod domain does not bind vinculin in vitro (Fig. 3.6F,G).
3.2.5 Blocking talin autoinhibition decreases talin turnover and promotes integrin adhesion complex assembly.

Defective morphogenesis could result from improper regulation of stability and turnover of integrin-mediated adhesions. To test this, we studied the adhesion dynamics exhibited by the autoinhibition defective talinGFP*E1777A using our previously established Fluorescence Recovery After Photobleaching (FRAP) protocol to examine the turnover of integrin and IAC components at MTJs in living Drosophila embryos and larvae [243]. FRAP analysis revealed that talinGFP*E1777A is more stable at MTJs than WT talinGFP (Fig. 3.4G). This data suggests that talin autoinhibition can modulate the turnover of integrin-based adhesion and that, specifically, preventing talin autoinhibition stabilizes the adhesion complex. Further examination of integrin-mediated adhesions in the AS supported this idea. We found that embryos rescued with talinGFP*E1777A exhibited greater integrin recruitment to the membrane of AS cells (Fig. 3.4H, J-K) and also to the leading edge of the epidermal cells that crawl over the AS (Fig. 3.4L-N). We also observed increased co-localization of talin and integrin in the AS (Fig. 3.4I-K). These observations are in line with reports in culture that expression of the talinE1770A autoinhibition mutant resulted in increased focal adhesion assembly [131].

3.2.6 The small GTPase Rap1 acts upstream of talin autoinhibition.

Our results indicated a link between autoinhibition and the regulation of the turnover and stability of integrin-based adhesions. The signaling molecules FAK and Rap1 have been implicated in such regulation [144, 263, 264] and we sought to see if either effector acts to regulate talin autoinhibition. Analysis of FAK failed to show any phenotypic parallels or
genetic interactions with talin autoinhibition; loss of FAK does not lead to defects in embryogenesis or disrupt viability [240]. Moreover, modulation of FAK activity does not impinge on turnover of either WT talin or the talinE1777A at MTJs (Fig. 3.7). We also tested the small GTPase Rap1, which has been implicated as part of a putative complex that localizes talin from the cytoplasm to adhesion complexes at the plasma membrane [138, 139] where it has been speculated autoinhibition can be relieved [74, 134, 135]. Our hypothesis was that increasing Rap1 activity would give rise to similar phenotypes to those observed in talinEGFP*E1777A-rescued embryos. To test this, we expressed a constitutively-active form of Rap1 (Rap1-Q63E; Rap1-CA) in the AS using the tissue specific Gal4 driver, c381; we observed similar DC defects to those seen with the autoinhibition defective talin (Fig. 3.8). Specifically, more than 60% of the Rap1-CA expressing embryos had open dorsal holes at the end of stage 15 with about 20% of the embryos failing to complete DC altogether (Figure 3.8A-B). We confirmed this delay using time-lapse imaging (Fig. 3.8A, Movie S3-4).

Furthermore, we found that colocalization of integrin and talin was increased in integrin-mediated adhesions of AS cells expressing Rap1-CA – this manifested itself as an increase in integrin signal at the membrane (Fig. 3.8C-F). We also tested the effect of expressing a dominant-negative form of Rap1 (Rap1-S17A; Rap1-DN) specifically in the AS and found that it also gave rise to DC defects. However, the Rap1-DN phenotype is different from that observed with the Rap1-CA in two ways: i) DC was not delayed but rather failed outright and ii) other morphogenetic problems, such as failed GBR, were observed (Fig. 3.9). Our data is consistent with previous work showing that expressing either Rap1-CA or Rap1-DN in the fly epidermis impairs DC, though the severity and range of phenotypes observed was different.
Altogether, these results indicated that Rap1 modulates integrin adhesion in the AS and is required for DC.

In addition to regulating integrin recruitment to the membrane in the AS, we also found that Rap1-CA increased the recruitment of talinGFP to MTJs (Fig. 3.8G). Therefore, we predicted that Rap1 might also regulate IAC turnover. FRAP analysis of talinGFP dynamics at MTJs revealed decreased turnover upon expression of Rap1-CA in the muscle (Fig. 3.8I). In comparison, expression of Rap1-DN elicited the opposite effect: turnover of talinGFP increased (Fig. 3.8J). To test whether Rap1 conferred its effect upstream of talin autoinhibition, either Rap1-DN or Rap1-CA were expressed in the presence of the talin autoinhibition mutant, talinGFP*E1777A. We found that Rap1-CA did not affect talinGFP*E1777A recruitment (Fig. 3.8H) and that neither Rap1-CA nor Rap1-DN modulated talinGFP*E1777A turnover (Fig. 3.8K,L). These results suggest that active Rap1 increases talin recruitment to and stabilization at cell-ECM adhesions, and that this effect occurs upstream of talin autoinhibition.

3.2.7 The Drosophila RIAM homolog, pico, acts upstream of talin autoinhibition.

It has been shown that the MRL-family protein RIAM links membrane targeting sequences in Rap1 to talin, thereby recruiting talin to the plasma membrane which leads to activation of integrin and enhanced adhesion [139, 266]. In general, the functions assigned to RIAM, including recruiting talin to the membrane and promoting stable adhesions are similar to those obtained by the relief of autoinhibition [138, 140, 266, 267]. Comprehensive analyses of the embryonic role of the Drosophila RIAM homolog, pico, are precluded at this time because the original loss of function allele has been lost. To circumvent this problem and to
test whether RIAM may also be involved in Rap1-dependent regulation of talin autoinhibition in the fly, we developed alternative approaches to modulate Pico/RIAM levels in the embryo. First, we used a minimal RIAM-Rap1 chimera (Fig. 3.10A; “RIAM30-CAAX”) comprised of the first 30 amino acids of human RIAM, which contains a talin binding site, and the membrane-targeting CAAX sequence of Rap1a, that was previously shown to be sufficient to activate integrins in CHO cells [139]. We found that expression of RIAM30-CAAX in the AS leads to delays in dorsal closure: approximately 80% of embryos exhibited open dorsal sides at the end of stage 15 (Fig. 3.10B-C). Furthermore, we found that RIAM30-CAAX induced increased recruitment of talinGFP to the membrane (Fig. 3.10D), and that the turnover dynamics of talinGFP decreased (Fig. 3.10G). The phenotypes conferred by increasing pico/RIAM via RIAM30-CAAX closely resembled those elicited by both the talinGFP*E1777A mutant and Rap1-CA suggesting that pico/RIAM could play a similar role in regulation of talin function. The ability of a human protein chimera to work as well as it does in flies illustrates the conservation of this system throughout evolution. Second, using an RNAi-induced knockdown of pico in the muscles, we found that the turnover of talinGFP increased (Fig. 3.10F,H), recapitulating the observed effect of expressing Rap1-DN. Importantly, neither the recruitment of talinGFP*E1777A to the membrane (Fig. 3.10E) nor the turnover dynamics of talinGFP*E1777A changed upon modulation of pico/RIAM (Fig. 3.10I-J), indicating that, like Rap1, Pico/RIAM modulates talin behavior via an autoinhibition-dependent mechanism. We propose that Rap1 and RIAM act upstream of talin to relieve autoinhibition; this promotes its recruitment to sites of adhesion where it forms a stabilizing link between integrins and the cytoskeleton (Fig. 3.10K). Our results also support the notion that a non-autoinhibited talin molecule can be recruited independent of Rap1/RIAM activity.
3.3 Discussion

Overall, this study identifies an important role for the regulation of talin function through autoinhibition. Failure to autoinhibit talin impairs morphogenetic processes but this is not due to defects in integrin-mediated attachment to the ECM or in the assembly of the adhesion complex. Thus it is unlikely that the E1777A mutation blocks integrin-mediated Cell-ECM attachment in a dominant negative fashion. An alternative explanation for the phenotype is that the E1777A mutant behaves like a gain of function allele of talin, and that the morphogenetic defects we observe are due to too much rather than too little adhesion. This would not be the first time such a phenomenon has been observed, for example overexpression of integrins in either the wing or the muscle gives rise to phenotypes identical to those found in integrin null mutants [235, 268]. How could the E1777A mutation give rise to stronger adhesion? We show that this mutation enhances the recruitment and co-localization of talin and integrin at sites of adhesion. Importantly, we show that the E1777A mutation effectively reduces talin turnover at sites of adhesion. Indeed, our data fits with a gain-of-function model: blocking talin autoinhibition leads to increased integrin-mediated adhesion, and this impairs morphogenetic processes that require cyclic adhesion assembly and disassembly. Further consistent with this model is the observation that adhesion at MTJs, a non-morphogenetic context, is not perturbed upon blocking autoinhibition of talin. We cannot exclude the possibility that E1777A may confer its effect on talin function through a means other than disruption of autoinhibition. Encouragingly, however, our homology modeling and NMR analyses strongly suggest that the fly protein behaves much as the mammalian homolog does.
How does preventing autoinhibition stabilize integrin-mediated adhesion? We show that autoinhibition regulates talin recruitment to adhesions through a RIAM-Rap1 dependent mechanism. Interestingly, the E1777A autoinhibition mutant talin is more strongly recruited to adhesions than WT talin; this enhanced recruitment occurs independent of RIAM-Rap1 activity. Thus, it is possible that constitutively relieving autoinhibition works to stabilize and promote adhesion by enhancing recruitment of the talin molecule to adhesions, thus bypassing the need of the RIAM-Rap1 pathway for recruitment. At the membrane, adhesion strengthening may occur via talin’s scaffolding function, as talin can interact with multiple components of the IAC, and these interactions may increase and/or change when talin assumes a more extended conformation. Another possibility, consistent with structural studies, is that relieving autoinhibition frees up the FERM/IBS-1 domain of talin such that it can activate integrins [108, 130]. We would predict that mutations in talin that block IBS-1-mediated integrin activation would lead to more dynamic adhesions, and this is indeed what was observed [260]. According to the model we envision, talin recruitment is determined by the sum of interactions that a single molecule can make with other IAC components at any one time. For example, the autoinhibited form of talin relies on Rap1/RIAM for efficient recruitment, even though it may still bind integrin through its free IBS-2 domain [9]; both mechanisms may contribute to targeting of talin to adhesions. We speculate that relieving autoinhibition makes the IBS-1 available, as well as the many other binding sites for IAC components that are found in the talin rod domain (e.g. vinculin binding sites), thereby substantially increasing the number of possible interactions that can lead to talin recruitment to the IAC.
There are likely multiple avenues leading to relief of talin autoinhibition. Recent super-resolution studies provided elegant evidence that autoinhibition is primarily relieved within adhesion complexes [134], implicating the need for a mechanism to specifically recruit autoinhibited talin to adhesions. Here we show that forcing talin to remain in an open, non-autoinhibited conformation gives rise to very similar phenotypes as activating the RIAM-Rap1 pathway. Based on the results obtained by us and other groups [133], 14, 25, 27, [141, 266], [102], we propose that RIAM-Rap1 brings autoinhibited talin to the membrane where autoinhibition can subsequently be relieved, possibly through electrostatic interactions with the membrane/PIP2. RIAM-Rap1 has a previously established role in mediating the recruitment of talin to sites of adhesion, but recently, it has been demonstrated that the requirement for RIAM-Rap1 is context-dependent. Structural and biochemical studies have revealed that the binding of talin to either RIAM or vinculin is mutually exclusive and likely dependent on force [102]. Moreover, in cell culture, vinculin-stimulated integrin activation is RIAM-Rap1 independent raising the possibility that more mature adhesions might not need RIAM-Rap1 to promote talin activation in this case [141]. Along similar lines, we demonstrate that RIAM-Rap1 activity is dispensable for recruitment of a non-autoinhibited talin molecule.

In summary, our results suggest that talin autoinhibition confers a switch through which fine control of integrin-mediated adhesion can be exerted in vivo. Our findings also reveal RIAM-Rap1-mediated regulation of integrin adhesion to be an important modulator of morphogenesis and provide evidence for an autoinhibition-based pathway for control of talin function through RIAM-Rap1. Furthermore, this study exemplifies how subtle tuning of adhesion complex composition and stability elicits different adhesive functions and cellular behaviors during development.
3.4 Materials and Methods

3.4.1 Molecular biology and homology modeling.

The generation of talinGFP is previously described [243]. To make pUbi-talinEGFP\*E1777A mutant construct, pBS-talinGFP was mutated using the QuikChange Lightning mutagenesis kit (Stratagene) and the following mutagenesis primers (only forward is shown, change indicated in bold): 5’-cccgttcgggtggtggcaagtgccattactct-3’. The talinGFP\*E1777A cassette was sub-cloned into the pUbi63E vector using a strategy similar to that used to generate the WT talinGFP construct [243].

To generate Rap1-CA (Rap1\textsuperscript{Q63E}) and Rap1-DN (Rap1\textsuperscript{S17A}), full-length \textit{Drosophila} Rap1\textsuperscript{WT} cDNA was PCR-cloned into the pCR8/GW/TOPO Gateway Entry Vector (Life Technologies, Grand Island, NY) by TOPO TA cloning. This entry vector served as the template for making Rap1\textsuperscript{Q63E} and Rap1\textsuperscript{S17A} by site-directed mutagenesis (QuikChange, Agilent Technologies, Santa Clara, CA). Rap1 mutant constructs were then recombined into \textit{Drosophila} UASp expression vectors modified for Gateway cloning, using Gateway vectors provided by Terence Murphy (Carnegie Institution for Science, Baltimore, MD). Expression vectors allowed for either a GFP or HA protein fusion at the N-terminus.

mCherry-RIAM30-CAAX (gift of James Lim, CFRI, Vancouver, Canada) was subcloned into a UAST-attB expression vector for fly transgenesis. All transgenic flies were generated by microinjection of fly embryos (BestGene, Chino Hills, CA) with the constructs introduced to the fly genome in a non-site directed way (talin and Rap1 constructs) or by phi31 integrase-mediated transgenesis (RIAM30-CAAX).
The homology-created fly structure of talin helices 37-41 (residues 1655-1826) was created using the program MODELLER [269]. Superposition, RMSD calculations and image rendering in figures 1B, 1C and 1D were performed in PyMol (Schroedinger, Inc.).

### 3.4.2 Protein expression and purification.

Fly talin F2F3 was amplified from talin cDNA and 1662-1828 (R9) was amplified from the *rhea* locus. Each construct was cloned into pLEICS-01 (N-HIS$_6$ TEV). Constructs were expressed in E. coli BL21 Star (DE3) cultured either in LB, or for preparation of 15N-labeled samples, in minimal media containing 1 g of 15N-ammonium chloride per liter. For the gel filtration and NMR experiments, GST-tagged F2F3 (residues 204-409) talin constructs were purified using glutathione sepharose resin (GE Healthcare) and eluted by TEV cleavage. His-tagged F2F3, R9 (residues 1662-1828) and R9 E1777A were purified on HisTrap HP column (GE Healthcare) and after TEV-cleavage of the tag, by ion exchange. Protein concentrations were determined using extinction coefficients at 280 nm.

### 3.4.3 Vinculin binding.

Analytical gel filtration chromatography using a Superdex-75 (10/300) GL (GE Healthcare) was used to assay binding of the talin R9 domain to the vinculin Vd1 domain. The two domains were incubated together at either 25oC or 42oC for 30 min prior to gel filtration. The column was pre-equilibrated and eluted with 20 mM Tris pH 8.0, 150 mM NaCl and 2mM DTT at a flow rate of 0.8 ml/min at room temperature.
3.4.4 NMR spectroscopy.

Proteins were prepared in 20 mM sodium phosphate pH 6.5, 50 mM NaCl, 2 mM DTT with 10% (v/v) 2H2O. NMR spectra were obtained at 298K using a Bruker AVANCE AVII 800 spectrometer equipped with CryoProbes.

3.4.5 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^{79a} allele and the Dominant Female Sterile technique[254]. Females of the genotype yw, hs-Flp/+; pUbi-talinGFP*WT or E1777A/+, rhea^{79a}, FRT2A/OvoD1, FRT2A were subjected to a heatshock-regime during the larval stages to generate 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 males. Embryos without the fluorescent balancer were selected for analyses. For all FRAP experiments, talinGFP constructs were heterozygous and expressed in a w^{1118} background. In the case where UAS-driven transgenes were utilized, comparable controls were taken from flies expressing the UAS-transgene, but without the mef2-Gal4 driver. RapCA was driven in the amnioserosa using the c381 driver or the LP1-Gal4 driver (gifts of Nick Harden).

3.4.6 Embryo staging and confocal immunofluorescence imaging.

For all experiments assessing dorsal closure, timed collections were performed to obtain a range of embryos from stage 12-13 (for live imaging), and stages 12-17 for phenotypic quantification of dorsal holes. Since gut morphology was not a consistent or
reliable indicator of stage in talin mutants, we used multiple criteria to stage our embryos based on the standard staging markers described in The Atlas of Drosophila Development [270]. The staging criteria used are as follows:

Stage 13 - germband-retraction complete, posterior spiracles apparent
Stage 14 - formation of posterior and anterior canthi
Stage 15 - completion of dorsal closure and re-epithelization, MTJs become prominent, initial formation of epidermal specializations (trichomes), heart-shaped gut
Stage 16 - MTJs fully formed, re-epithelialization of the epidermis at the dorsal midline is complete; multi-constricted gut, onset of muscle contraction
Stage 17 - looped gut, denticle belts formed, mouth hooks visible

Our staging was assisted by the fact that all of our talin-transgene-rescued embryos ubiquitously expressed talinGFP which labels most structures. In the case of embryos that completely failed in DC, the body plan was too disrupted to judge whether embryos were stage 16 or stage 17. For Rap1-CA and Rap1-DN experiments, we performed our phenotypic analysis on fixed embryos stained with rhodamine-conjugated phalloidin to visualize morphology for staging.

3.4.7 Embryo fixation and image analysis.

Embryos were fixed and stained according to standard protocols. The following antibodies were used in our analysis: rabbit anti-talin (1:500), mouse monoclonal anti-βPS-integrin (1:50; DSHB), rat anti-αPS2-integrin (1:200, 7A10), mouse anti-tiggrin (1:1000; Liselotte Fessler, UCLA), and rabbit anti-paxillin (1:1000; [239]). 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 a 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 1.0µm confocal sections. For all amnioserosa micrographs, z-stacks were assembled from 3-5 0.6µm confocal sections, just under the apical surface.

For intensity traces across MTJs, the ImageJ plot profile tool was used to determine the average signal intensity across the boxed area indicated on the images. Each channel was independently normalised from unprocessed grey-scale images so that the peak intensity of each channel across the area of interest was set as 100% and the lowest intensity was set to be 0%.

For quantification of βPS-integrin staining in the amnioserosa, all images were taken at identical settings and signal at the cell periphery was quantified according to following formula:

\[
\text{Corrected Fluorescence Intensity} = (\text{mean fluorescence of cell periphery})*(\text{area of measured signal}) – (\text{mean background fluorescence})*(\text{area of measured signal})
\]

For colocalization analysis of talin and integrin in the amnioserosa, the colocalization feature in Olympus Fluoview software was used to determine Pearson Correlation Coefficients. At least 5 cells in 5 different embryos were used for analysis of β-integrin fluorescence and colocalization in each genotype.
Talin recruitment was quantified as previously described [235, 260] with some modifications as follows: stage 17 embryos were dechorinated in 50% bleach and mounted on a glass slide in PBS. Single confocal images of 1um thickness were collected from 8-10 different confocal planes and the ratio of the MTJ-localized talin to the cytoplasmically localized talin was measured in each individual plane and then averaged. This was performed for at least 10 different MTJs for each genotype.

3.4.8 Time-lapse imaging.

For time-lapse movies, stage 13 embryos were dechorinated, glued to a coverslip, dorsal side down, and mounted in Halocarbon Oil 27. Coverslip bridges were used to prevent tissue compression. Using a 40x lens, about 25-30 2.0µm confocal sections were collected at 2-min intervals for an eight-hour time periods using a 473nm laser at room temperature. Several movies were taken of each genotype. Animations were assembled and processed using ImageJ.

3.4.9 FRAP

Stage 17 embryos were collected and prepared for FRAP as described previously [243]. 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 405 nm laser at 30% 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.

3.4.10 qPCR.

For the quantitative real-time PCR (qPCR) data shown in supplementary material Fig. S2C, total RNA was isolated from whole flies using TRIzol (Invitrogen) and treated with DNase (Fermentas). A total of 1000 µg total RNA was converted into cDNA using the qScript cDNA Synthesis Kit (Quanta Biosciences). Subsequently, qPCR was performed using the PerfeCTa SYBR Green FastMI ROX kit (Quanta Biosciences). As all talin transgenes were GFP-tagged, we could to quantify transgenic mRNA transcript levels by assaying GFP expression using the primer pair: 5’- GCAGAAGAACGGCATCAAGGT-3’ and 5’-ACGAACTCCAGCAGGACCATG-3’. GAPDH mRNA levels were assayed as an internal control using the primer pair: 5’- AAAGCGGCAGTCGAATAGC-3’ and 5’-GACATCGATGAAGGGATCGT-3’. Expression changes were determined by using the comparative Ct method for relative quantitation.

3.4.11 Western blots.

Adult thoraces of wild-type flies expressing TalinGFP and TalinGFP*E1777A mutants were dissected in 50mM Tris, 1mM EDTA, 150mM NaCl, 0.5% Triton, 50% Glycerol and EDTA-free complete protease inhibitor cocktail (Roche) and incubated overnight at -20°C. Samples were homogenized with a mechanical pestle in 50mM Tris, 1mM EDTA, 150mM NaCl, 0.5% Triton and EDTA-free complete protease inhibitor cocktail (Roche) and
cleared by centrifugation at 13,000 rpm for 30 min at 4°C. After the addition of SDS sample buffer, samples were heated for 5 minutes at 100°C and separated on a 6% Tris-glycine SDS-polyacrylamide gel before performing a Western Blot. The membrane was blocked in 5% milk for 20 m and incubated with rabbit anti-talin antibody (1:2000, gift of Nick Brown (Gurdon Institute, Cambridge, UK); [200]) or mouse anti-alpha tubulin antibody (1:2000, Sigma) and ECL anti-rabbit or anti-mouse HRP-Conjugated secondary antibody (1:5000, GE) and visualized with ECL Western Blotting Plus Detection system (GE). Quantification of blots was performed using ImageJ (NIH, Bethesda, MD). Westerns were performed in a wild-type background to facilitate comparison of the expression of our GFP-tagged transgenic talin to the untagged endogenous protein. This approach provides important confirmation that the transgenes are sufficiently expressed.
3.5 Chapter 3 Figures

**Figure 3.1. The mechanism of autoinhibition is structurally conserved between mammals and Drosophila.**

(A) Cartoon schematic of talin autoinhibition. (B-D) The NMR structure\[108\] of mouse R9 (B) and our homology-predicted model of fly talin R9 (C). Critical residues for F3-rod binding are highlighted in red. (D) Superposition of the mouse NMR structure (yellow) and the homology-modeled fly structure (blue). (E) Coomassie-stained SDS-PAGE gel showing that purified recombinant WT and E1777A fly R9 domains exhibit similar electrophoretic mobility at the expected molecular weight. (F) \(^1\text{H},^{15}\text{N}-\text{TROSY-HSQC}\) spectra of 150 \(\mu\text{M}\) \(^{15}\text{N}\)-labeled WT talin R9 (blue) and R9 E1777A (red). The R9 E1777A mutant shows a well dispersed NMR spectrum similar to that of the wildtype R9 indicating that the mutation does not affect the tertiary structure of the domain. (G) A \(^1\text{H},^{15}\text{N}-\text{TROSY-HSQC}\) spectra of 25 \(\mu\text{M}\) \(^{15}\text{N}\)-labeled fly talin F2F3 alone (blue) or in the presence of the talin rod R9 domain (red). In the presence of R9, some of the peaks have shifted and broadened (indicated by asterisks) compared to the spectra of the free F2F3 providing evidence of a direct interaction between fly F2F3 and R9.
**Figure 3.2. Species alignment of domains required for talin autoinhibition.**
Alignment of Human (Hs Tln1), Mouse (Mm Tln1), Chicken (Gg Talin-1), and fly (Dm Rhea) talin FERM F3 (A) and R9 (B). Arrowheads bracket the specific, structurally defined regions. Red asterisks: residues implicated in autoinhibition; green asterisks: residues implicated in other F3-dependent interactions.
Figure 3.3. Disruption of the autoinhibitory intramolecular interaction between the talin FERM and the talin rod leads to morphogenetic defects including delayed dorsal closure. (A-F) Late stage talin-null embryos stained for integrin (green in A-D) and F-actin (magenta in A-D) were scored for phenotypes in the morphogenetic processes DC (J,L; asterisk in J demarcates open dorsal hole) and GBR (K,M; arrowhead in K shows un-retracted tail). Embryos were rescued with talinEGFP (H) construct or the talinEGFP*E1777A autoinhibition mutant construct (J-K). (G-H). Talin-null embryos rescued with either talinGFP or talinEGFP*E1777A were scored for dorsal holes at stage 13-17 (see Experimental Procedures). Images from time-lapse movies of WT-rescued embryos (O, top) or E1777A mutant embryos (O, bottom) expressing talinGFP*E1777A and undergoing DC at the indicated time-points.
Figure 3.4. Talin recruitment and adhesion dynamics are altered by the E1777A autoinhibition mutation.

(A-C) qPCR (A) and Western blot data (B-C) for talinEGFP (orange), autoinhibition mutant talinEGFP*E1777A (purple) and endogenous untagged talin (black). Talin was detected with a polyclonal antibody raised to the C-terminus (see [200]) and Westerns were done in a wild-
type background. (D-F) The recruitment of talinGFP (D,E) and talinGFP*E1777A (D,F) at MTJs (C, p<0.01). (F) Fluorescence recovery curves of talinGFP (orange) and talinGFP*E1777A (purple) obtained from FRAP experiments on embryonic MTJs, *p<0.001. (G-N) βPS-integrin signal was quantified at the lateral membrane of AS cells (H) and the leading edge of the epidermis (L) and colocalization of talin (magenta in J’,K’) and βPS integrin (green in J’-K’,M’-N’; black in J’-K’,M’-N’) was measured at the lateral membrane of AS cells using Pearson correlation co-efficients (I; n>25 cells, *p<0.05, ***p<0.001). F-actin is shown in magenta in M’-N’ to highlight the leading edge.
Figure 3.5. Talin autoinhibition is not required to maintain muscle attachment to the ECM or for IAC assembly at MTJs.
(A-C) Confocal z-stacks of muscles of talin null embryos (A), WT-rescued embryos (B), and E1777A rescued embryos (C) stained with phalloidin to label F-actin to show muscle morphology. (D) Penetrance of muscle attachment defects in stage 17 embryos for each of the indicated genotypes. (E) Recruitment of actin to muscle ends in WT-rescued (orange) and E1777A-rescued (purple) talin mutant embryos. (F-K) Confocal z-stacks of MTJs in talin-null embryos rescued with either talinGFP (F,H,J) or the talinGFP*E1777A autoinhibtion mutant (G,L,K) labelled for tiggrin (grey in F,G; magenta in F''', G'''), α-integrin (grey in F',G',H',I',J',K'; green in F'',G''; magenta in H',I',J',K'), talin (grey in H-I, green in H''-I''), and paxillin (grey in Q, R; green in Q'', R''). Average intensity profiles of tiggrin and α-integrin (F'''-G'''), α-integrin and talin (H'''-I'''), and α-integrin and paxillin (J'''-K'''') across the widths of the boxed areas indicated in the corresponding images shown in F''-K'''.
Figure 3.6. Talin autoinhibition does not affect vinculin recruitment to sites of integrin function in Drosophila. (A-D) Confocal z-stacks of MTJs in embryos expressing talinGFP (A) or talinGFP*E1777A (B) and stained for vinculin (green in A-B; grey in A’-B’) and talinGFP (red in A-B). Vinculin recruitment (C) and colocalization of vinculin and talin (D) were measured at MTJs. (E) Stage 14 embryo stained for F-actin (magenta in E; grey in E’) and vinculin (green in E; grey in E’’). Vinculin is not expressed in the amnioserosa. (F) Sequence alignment of Helix 40 of mouse (top) and fly (bottom) talin. The consensus vinculin binding sequence is shown below. (G) Analytical gel filtration of R9 mixed with vinculin Vd1 indicating that vinculin and R9 do not bind.
Figure 3.7. Loss of FAK does not perturb the turnover dynamics of talinGFP or talinGFP*E1777A at MTJs.

(A) Fluorescence recovery curve of talinGFP in a WT background and in a FAK null background (FAK<sup>cg1</sup> allele; see [240]). (B) Fluorescence recovery of autoinhibition-impaired talin, talinGFP*E1777A in a WT background and in a FAK null background.
Figure 3.8. Rap1 functions upstream of talin autoinhibition during morphogenesis.
(A) WT embryos and embryos expressing Rap1-CA in the AS were scored for openings in the dorsal epidermis at stage 13-17. (B) Images from time-lapse movies of control embryos (top) or embryos expressing Rap1-CA in the AS (bottom) undergoing DC at the indicated time-points. (C-F) β-integrin signal localized at the lateral membrane of AS cells was quantified (C) and colocalization of talin (magenta in E’,F’) and β-integrin (black in E,F; green in E’,F’) was measured at the membrane of AS cells using Pearson Correlation Co-efficients (D; n>25 cells, *p<0.05,***p<0.001). (G,H) The recruitment of talinGFP (D) and talinGFP*E1777A (E) to MTJs was measured in control embryos (orange in G; purple in H) and embryos expressing Rap1CA (pink; **p<0.01). (H-K) FRAP experiments were performed on talinGFP (I,J) and talinGFP*E1777A (K,L) to determine the effect of expressing either Rap1-CA (I,K) or Rap1-DN (J,L) on the mobility of talin at MTJs.

Figure 3.9. Expression of Rap1-DN in the amnioserosa leads to defects in morphogenesis distinct from the phenotype of expression of Rap1-CA.

(A) Stage 14 embryo expressing Rap1-DN in the AS exhibiting both failed dorsal closure (asterisk) and failed germian retraction. (B) Dorsal view of a stage 15 embryo exhibiting a failure to close the dorsal epidermis. (C) WT embryos and embryos expressing Rap1-CA in the AS were scored for openings in the dorsal epidermis at stage 13-17. Rap1-DN expressing embryos exhibited a partially penetrant failure in DC, but a delay in dorsal closure was not observed as a similar proportion of embryos had completed DC at stage 15 as stage 16/17 (Compare to Figs. 1O, 3B, 4B).
Figure 3.10. Pico/RIAM functions upstream of talin autoinhibition during morphogenesis.
(A) Schematic diagram of RIAM30-CAAX (B) WT embryos and embryos expressing RIAM30-CAAX in the AS were scored for openings in the dorsal epidermis at stage 13-17. (C) Stage 15 embryo with an open dorsal hole (arrowhead) stained for amnioserosa (green), integrin (red), and F-actin (blue). (D-E) The recruitment of talinGFP (D) and talinGFP*E1777A (E) to MTJs in control embryos and embryos expressing RIAM30-CAAX. (F) To provide evidence of pico knockdown based on the previously described pico phenotype [271], we measured adult body size of control embryos and embryos expressing picoRNAi.
under the control of a ubiquitous driver. (G-J) FRAP experiments were performed on talinGFP (G,H) and talinGFP*E1777A (I,J) to determine the effect of expressing either RIAM30-CAAX (G,I) or picoRNAi (H,J) on the mobility of talin at MTJs. (M) Model for the role and regulation of talin autoinhibition. RIAM-Rap1 acts to localize autoinhibited talin to integrin-mediated adhesions where autoinhibition can be relieved by electrostatic membrane interactions. This mechanism promotes stable adhesion, thus down-regulating cell motility required for morphogenesis.
CHAPTER FOUR: The Talin Head is Required for Adhesion Complex Assembly and Stability

4.1 Introduction

Elucidating how cell-ECM adhesion is regulated in vivo is of central importance to clarifying our picture of how tissue structure is formed and maintained. Integrins themselves lack the capacity for catalytic activity, but integrin regulation is efficiently achieved via interactions with both extracellular ligands and intracellular binding partners via either “outside-in” or “inside-out” signaling mechanisms. Integrins are known to regulate their function in two key ways: (1) by conformational control of integrin ligand affinity in a process known as integrin activation whereby the heterodimer switches between a bent, inactive state and an extended, active state; (2) by altering integrin avidity or “clustering” by making lateral interactions in the plasma membrane with other integrin heterodimers. A key mediator of both these processes is the cytoskeletal linker protein talin, which forms a direct structural link between β-integrin cytoplasmic tails and the actin cytoskeleton. The N-terminus of talin contains a non-canonical, extended Band 4.1/Ezrin/Radixin/Moesin (FERM) domain, comprised of subdomains F0, F1, F2, and F3 [105]. Talin-head binding to the membrane-proximal NPxY motif of the β-integrin cytoplasmic tail is well understood to induce inside-out integrin activation [69, 70]. Interactions of the talin F2-F3 sub-domains with the membrane proximal region of the β-integrin cytoplasmic tail and the plasma membrane induce a change in tilt angle of the β-integrin transmembrane domain [64, 256]. This angle change results in separation of the alpha and beta integrin subunits, which promotes extension of the
extracellular domain, and also facilitates the formation of lateral interactions with other integrins in the membrane. Talin has been shown to induce integrin clustering via unclear mechanisms [95, 96] and it has been further suggested that integrin-clustering is required for efficient talin recruitment [235]. In addition to regulating integrin activity and avidity, talin is also known to play many other crucial roles in adhesion scaffolding and dynamics, as well as in mechanotransduction.

Our previous work addressed the role of the integrin-talin head interaction during fly development by using a mutation that completely abolishes this interaction (R367A in fly; R358A in mouse). We discovered that disrupting the integrin-talin head interaction, attachment between integrins and their ECM ligands was compromised leading to tearing in the somatic musculature at myotendinous junctions (MTJs) [100]. However, in these studies, we could not separate between effects due to loss of integrin activation versus the need for a structural linkage between integrins and the talin head. Moreover, a cell-culture based study suggested that talin-head induced integrin activation might not occur in Drosophila, leading the authors to conclude this may be a result of underlying differences between Drosophila and vertebrate integrins [99]. A subsequent report demonstrated that the antibody raised to recognize active mammalian αIIbβ3 integrin (PAC-1 IgM) effectively provides a multivalent ligand, which would bind to multiple integrin heterodimers and thus induce receptor clustering, making it impossible to distinguish between the roles of talin in control of clustering versus activation [95]. New methods were developed to circumvent this problem and it was found that in the presence of a monovalent ligand, the talin head could not induce inside-out activation of αIIbβ3 integrin in CHO cells and in platelets [95]. In light of this finding, the relative contribution that talin-dependent integrin activation versus clustering
makes to the form and function of integrin-mediated adhesions remains an important outstanding question.

Here, we specifically address the role of integrin activation in the context of the developing fly embryo. Moreover, we aim to identify other functions of the talin head that play a role in integrin-dependent processes. Surprisingly, we find that the ability of the talin head to induce changes in integrin affinity did not significantly contribute to integrin function in the context of morphogenesis or tissue maintenance. Our results show that the major function of the talin head is to induce integrin receptor clustering, and to promote adhesion maturation. Furthermore, we uncover a novel function of the talin head conferred by specific inter-domain interactions, and demonstrate that this function is essential for integrin-dependent tissue development and maintenance.

4.2 Results

4.2.1 Integrin activation is not essential for fly embryogenesis

In mammalian talin, NMR studies revealed that disrupting the interaction between talin and the membrane proximal part of the β3 integrin cytoplasmic tail was sufficient to abrogate integrin activation, despite the fact that talin binding with more membrane-distal parts of the integrin cytoplasmic tail was not perturbed [73]. Since the residue that was mutated to disrupt the talin head-integrin membrane-proximal cytoplasmic interaction is conserved between flies (L334) and vertebrates (L325 in talin1, L328 in talin2), we could specifically test the effect of disrupting integrin activation during fly embryogenesis by making the equivalent mutation in fly talin: L334R (Fig. 4.1a).
Using the domainant-female sterile germline clone technique [254] (see Materials and Methods), we removed all endogenous talin from fly embryos and replaced it with ubiquitously expressed full length talin constructs, either wild-type (WT) talinGFP, or the integrin activation mutant, talinGFP*L334R. Surprisingly, we found that in comparison to talinGFP-rescued embryos (Fig. 4.1b), talinGFP*L334R-rescued embryos looked almost identical in that there were no obvious defects (Fig 4.1c). Closer analysis of integrin and ECM localization MTJs revealed that in contrast to the effect of the talinR367A mutation (Fig. 4.1e), and similar to WT talinGFP-rescued embryos (Fig 4.1d), tearing of integrins away from the ECM was not observed in embryos rescued with talinGFP*L334R (Fig 4.1f). We hypothesized that a possible explanation for this unexpected result was that in fly talin, the L334R mutation might not be sufficient to prevent integrin activation. To test this possibility, we expressed fly talin head constructs, either WT or L334R, in CHO cells and used a well established flow cytometry-based assay to quantify activation of αIIbβ3 integrins (Fig 4.1g). We found that L334R efficiently disrupted integrin activation. Based on this result, we conclude that talin-dependent integrin activation may be dispensable for fly development.

Upon closer examination, we found a few subtle defects. Firstly, we observed that recruitment of talinGFP*L334R to sites of integrin-mediated adhesion was less efficient than WT talinGFP (Fig. 4.1h). We observed this effect in the presence of the endogenous talin suggesting that the mutant protein is outcompeted by the untagged WT protein for integrin binding. Secondly, we used Fluorescence Recovery After Photobleaching (FRAP) to demonstrate that the dynamics of talinGFP*L334R were increased compared to WT talinGFP (Fig. 4.1i). This observation is consistent with the trends of our previous findings that completely blocking the talin head-integrin interaction also increases the turnover of talin at
myotendinous junctions [260]. In this case however, perhaps the change in talin turnover is not of sufficient magnitude to induce any deleterious functional effects. Taken together, these data show that while cell-ECM attachment was not significantly impaired, talin-induced activation of integrins does play a role in stabilizing talin at sites of integrin-mediated adhesion.

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

We sought to identify roles of the talin head other than integrin activation and integrin binding. To this end, we completely deleted the talin head and, using the same approach described above, replaced endogenous talin with a ubiquitously expressed talin head construct: headless-talinGFP. The resulting embryos displayed failures in multiple integrin-dependent processes that occur during fly embryogenesis (Fig 4.2). We examined two different integrin-dependent morphogenetic events that occur to shape the fly embryo: (1) germband retraction (GBR), which is required to reposition the tail at the caudal end of the embryo, and (2) dorsal closure (DC), which is a wound healing-like event that establishes a continuous layer of epidermis around the circumference of the embryo. In the complete loss of talin, 53.8% of embryos fail at GBR (Fig 4.2a,d), and 63.9% of embryos fail at DC (Fig. 4.2a,e). In comparison, 60% of headless-talinGFP rescued embryos failed in GBR (Fig. 4.2c,d), and 30.8% failed in DC (Fig 4.2c,e). This is in contrast to talinGFP-rescued embryos, of which 4.2% and 5% failed in GBR and DC, respectively (Fig 4.2b,d-e). These results indicate that the talin head is very important for mediating the functions of talin during morphogenesis. GBR is more strongly affected than DC.
Integrins mediate connections between adjacent muscle cells as well as to the overlying epidermis through an intervening ECM at specialized structures known as MTJs. In the absence of integrin or talin function, MTJs cannot support the force associated with muscle contraction, and thus muscles round up and detach from one another. 100% of animals homozygous for null alleles of talin or integrins exhibit this characteristic “myospheroid” phenotype (Fig 4.2a,f). We also found that 100% of headless-talinGFP-rescued embryos had defects in muscle attachment (Fig 4.2c,f). This result is unsurprising as it was previously found that mouse embryonic fibroblasts expressing only headless-talin were unable to support cell spreading [153].

4.2.3 The talin head is required to stabilize the integrin adhesion complex at MTJs.

Upon examination of headless-talinGFP fluorescence localization in stage 17 WT embryos, we found that enriched GFP fluorescence at MTJs was hardly detectable compared to WT talinGFP (Fig 4.2g). Interestingly, we found that in the absence of any endogenous talin, headless-talinGFP was well recruited and maintained at MTJs (Fig 4.2i-j) suggesting that in line with what we observed for the talinGFP*L334R mutant, the mutant protein was less able to compete with endogenous, untagged talin for recruitment (Fig 4.2i). We also found that the stability of headless-talinGFP was markedly decreased compared to WT talinGFP. FRAP analysis revealed that the mobile fraction of the mutant protein more than doubled compared to that of WT talinGFP (Fig 4.2h). These results suggest that in the absence of the talin head, although talin can still be recruited to integrins via talin rod-dependent interactions, the stability of talin at integrin-mediated adhesions is reduced.
We wondered if impaired talin stability might have consequences for the recruitment of other integrin adhesion complex components, especially since talin has been implicated as a master regulator of adhesion complex assembly in Drosophila. We found that integrin was normally localized and maintained at MTJs of talin null embryos rescued with headless-talinGFP compared to WT talinGFP rescues (Fig. 4.2i-j). However, in comparison, we found that the localization of other adhesion complex components including zasp and paxillin was strongly reduced at MTJs (Fig. 4.2k-n), although we did observe weak, highly variable recruitment in some embryos. These results indicate the talin head is critical for maintaining a functional adhesion complex, and provide a potential explanation underlying the strong muscle detachment phenotype we observed.

4.2.4 rhea17 encodes a hypomorphic talin protein which disrupts talin head function

To gain further insight into how the talin head carries out its essential functions, we turned our attention to a previously uncharacterized allele of talin, rhea17, originally uncovered in a screen for dominant enhancers of a hypomorphic integrin allele [200]. rhea17 encodes a talin protein containing a missense mutation in a conserved glycine, G340 (G331 in mammalian talin1, G334 in mammalian talin2), to a glutamate (G340E; Fig 4.3a). Phenotypic analyses of embryos homozygous for this mutation displayed severe defects that closely mirrored the effect of deleting the talin head (Fig 4.3b-c). Integrin-dependent morphogenetic processes GBR and DC were partially disrupted compared to heterozygous controls. Most strikingly, however, rhea17 embryos exhibited a fully penetrant muscle detachment phenotype. One possibility to explain the strong phenotype we observed was that the mutation compromises the stability of talin protein such that functional defects would arise from
insufficient protein levels of talin. However, an antibody raised against the C-terminal 534 amino acids of talin robustly detected signal corresponding to the expected localization pattern of talin indicating the molecule was transcribed and translated downstream of the genetic lesion (Fig. 4.3i-l). Thus, the phenotype incurred by the rhea$^{17}$ allele is a specific result of the G340E point mutation, not due to insufficient protein levels of talin.

Our analysis of rhea$^{17}$ revealed that the function disrupted by the G340E mutation did not interfere with the localization of the full length talin molecule to MTJs. Interestingly, we found that if we engineered this mutation into an isolated talin-head construct and expressed it specifically in the muscle under the control of mef2-Gal4, UASp-GFP-talin-head*G340E was not enriched at MTJs relative to what was observed for WT UASp-GFP-talin-head (Fig. 4.3d-f). Moreover, using a cell culture-based integrin activation assay, we found that talin-head*G340E failed to induce robust integrin activation compared to the WT control (Fig. 4.3g). This data suggests that the G340E mutant likely is unable to stably associate with β-integrin cytoplasmic tails and thus is functionally impaired.

Further analysis of rhea$^{17}$ embryos revealed that, similar to headless-talinGFP rescued embryos, IAC assembly and maintenance were also defective. In fact, while talin enrichment at MTJs exhibited a marginal increase between stage 16 and stage 17 (Fig. 4.3h-l), the levels of other IAC components localized to MTJs, including PINCH (Fig. 4.4a-e), pFAK (Fig. 4.4f-h and data not shown), and Paxillin (data not shown) were significantly decreased at stage 16 compared to control, and dropped dramatically in stage 17. Together, these data demonstrate that the G340E mutation in the talin head is sufficient to abrogate all the functions of the talin head.
4.2.5 The G340E mutation disrupts integrin clustering

The talin head is known to be important for mediating integrin clustering. To determine whether or not the G340E mutation impinged integrin clustering, we used a well-established in vivo assay to assess integrin clustering in the context of the imaginal wing disc epithelium. Integrins mediate adhesion between epithelial layers and form discrete puncta that colocalize with other adhesion complex components, including talin, on the basal surface of the epithelium [200]. In the absence of talin, these clusters do not form, indicating a role for talin in integrin clustering [200]. Interestingly, we found that clonal patches of homozygous rhea\(^{17}\) mutant cells also failed to form integrin clusters (Fig. 4.4i-j). Therefore, these results suggest this particular residue, G340, plays a critical role in talin head function to promote integrin clustering and stable IAC maintenance.

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

What is the specific effect of the rhea\(^{17}\) mutation? The fixed orientation of the F2-F3 domains has important implications for the ability of the talin head to activate integrins [64]. We have shown previously that a cluster of basically charged residues in the F2 domain, the membrane orientation patch (MOP) (Fig. 4.5a), plays 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 and multiscale molecular dynamics simulations suggest that this results in a \(\sim 20^\circ\) change in the tilt of the \(\beta\)-integrin transmembrane domain [256] leading to integrin activation via dissociation of the \(\alpha\)-integrin and \(\beta\)-integrin transmembrane domains (Fig. 4.5b).
The G340E mutant identified in our studies (corresponding to G331E in talin1, G334E in talin2) is located on the surface of F3 in its interface with F2 (Fig. 4.5a). In the wildtype this glycine allows the close packing of the two sub-domains. However, substitution for a glutamate, whilst having no effect on the ability of F3 to engaged the β-integrin (Figure 4.5c), inserts a charged carboxyl group into this close gap disrupting the fixed orientation of the F2 and F3 domains allowing them to move independently of one another. Significantly, this mutation means that the rotation of F2 to optimize the membrane interaction can occur without rotation of F3 and the resulting adjustment of the β-integrin transmembrane domain tilt angle (Fig. 4.5c). Therefore the G340E mutant can still engage the integrin via the F3 domain but it cannot induce the necessary conformational changes in integrins required to sustain stable adhesions.

4.3 Discussion

Our studies have provided for the first time, a comprehensive in vivo assessment of the functions of the talin head. We used targeted mutations in transgenically expressed rescue constructs, as well as analysis of a previously uncharacterized mutation in the native talin locus to identify specific mechanisms of talin function in the developing fly embryo. Our analyses have revealed three key insights: (1) talin-dependent integrin activation is dispensable for integrin function in flies; (2) the major function of the talin head is to promote integrin clustering and adhesion maturation; (3) long-term maintenance of the integrin adhesion complex may depend on a novel, previously undescribed mechanism involving F2-F3
interactions; (4) deletion of the talin head, or disruption of the F2-F3 interaction, leads to severe defects in integrin-mediated processes during fly embryogenesis.

Attempts to delineate the role of control of integrin affinity (activation) versus avidity (clustering) have given rise to mixed conclusions about the relative contribution of each mechanism. An elegant study using negative-stain EM demonstrated increased extension of single integrin heterodimers embedded in lipid bilayer nanodiscs upon addition of talin head indicating that talin can induce conformational changes independent of receptor clustering; however, this population of extended heterodimers represented a relatively small proportion of the total number of integrins counted \[71\]. Our study suggests that the talin head controls integrin-mediated adhesion primarily by regulating integrin clustering. In complete absence of the talin head, or in the case of the G340E \textit{rheal} \[17\] mutant, integrin receptor clustering cannot occur and thus a vital scaffold for the rest of the adhesion complex is lacking leading to extreme deficits in integrin function. Since integrin clustering would create a more concentrated platform for adhesion complex formation and maintenance, it is reasonable to postulate that integrin clustering promotes robust IAC assembly and adhesion maturation. In line with this prediction, we observed progressive loss of adhesion complex components from MTJs when the talin head was deleted or functionally compromised by the G340E mutation. In the context of a multicellular tissue, it is unlikely that integrins would ever come across truly multivalent ligand, so it is not unreasonable to suggest that integrin clustering could be the driving mechanism underlying adhesive contact formation.

We do not aim to suggest that modulation of integrin-ligand affinity is not important in \textit{Drosophila}. In fact, our own previous work argues against this since preventing binding between integrin cytoplasmic tails and the talin head resulted in muscle detachment due to
weakened linkage between βPS-integrins and the ECM [100]. It may be that in the case of the L334R mutation, which was designed to specifically abrogate integrin activation, and did indeed prevent integrin activation of αIIbb3 integrins, other molecules, such as kindlins or Zasp, can compensate for loss of talin and contribute to integrin affinity regulation in *Drosophila* [79, 272]. However, if the talin head is completely unable to bind integrin, as was the case with the talin*R367A* mutation used in our prior study, the co-activating action of these factors may be insufficient and thus the integrin-ligand attachment is weakened leading to the observed phenotype. Nonetheless, this was a considerably milder phenotype than was initially predicted. At the MTJ, outside-in signaling could be the major factor regulating integrin affinity since some ECM molecules are recruited to MTJs independent of integrins [255]. Another possibility previously put forward by Helsten and colleagues is that there may be evolutionary divergences between vertebrate and Drosophila integrins such that they are not regulated via the same mechanisms [99]. However, our homology modeling failed to reveal any obvious differences in how talin head-beta integrin tails dock with one another between the two species (data not shown). There are likely to be cell-type and/or microenvironment-specific conditions under which talin predominantly controls integrin affinity over avidity and vice versa.

Talin is known to make many intramolecular interactions, some of which promote talin activity while others are thought to inhibit [101, 108, 130]. For example, we previously showed that intramolecular interactions between the talin FERM and the talin rod were essential for negatively regulating talin function during epithelial morphogenesis [273]. However, this current work reveals an unforeseen interaction between F2 and F3, which seems to be absolutely required for the activity of the talin head. Many biochemical studies have
predicted a role for precise regulation of the talin head orientation relative to the plasma membrane [62, 64, 256], and based on our work, we propose that the apposition between F2 and F3 is required to facilitate optimal interaction between F2 and the plasma membrane, which allows the talin head to be stably maintained in integrin-mediated adhesions.

If the explanation underlying the rhea\textsuperscript{17} phenotype were simply that the head could no longer bind integrin, we would expect to observe in rhea\textsuperscript{17} embryos a very similar phenotype to what we previously observed when we abrogated the talin-head interaction using the talin*R367A mutation. Although both mutations blocked the ability of the talin head to be independently recruited to adhesions and gave rise to increased IAC dynamics, the two phenotypes were, in general, vastly different from one another. Unlike rhea\textsuperscript{17}, talin*R367A did not interfere with integrin clustering or adhesion complex maintenance, and failed to give rise to any of the severe morphogenetic defects conferred by the rhea\textsuperscript{17} allele. Therefore, we believe we have identified a previously unexplored function for the talin head, which does not depend upon its integrin-binding capacity.

Our results point to a role for the talin head and F2-F3 domain organization in adhesion maturation. How might this occur? An important functional aspect of adhesions at MTJs is the ability to sense force and remodel in response to changes in tensile forces, which inherently increase during animal development [244]. Talin unfolds in response to force to expose binding sites for its binding partners [147]. This is one possible mechanism through which talin might promote adhesion reinforcement. This phenomenon, however, is likely to be dependent on the presence of the whole molecule as predicted by our data and by Zhang et al, who demonstrated that the full-length talin molecule is required for a cell to exert traction on the substrate [153]. Consistent with our results, only the full-length talin molecule was able to
restore normal cytoskeletal organization and focal adhesion assembly in mouse embryonic fibroblasts depleted of endogenous talin [153]. Neither expression of the talin rod nor the talin head alone was sufficient to rescue this function [153]. In embryos in which talin head function has been disrupted, either by complete deletion or by the rheal\textsuperscript{17} mutation, we observe a pronounced loss of IAC components between stages 16 and stage 17, a period of several hours of growth in which sarcomeres form and muscle contraction begins. We anticipate that as increased force is exerted on integrin-mediated adhesions at MTJs, the full-length, intact talin molecule is required to facilitate adhesion reinforcement. If the stability of the talin head at adhesions is compromised (as in rheal\textsuperscript{17}) or deleted altogether (as in headless-talinGFP), this reinforcement cannot occur leading to the disintegration of MTJs and subsequent muscle detachment in stage 17.

In summary, our results provide insights into how integrins are regulated under physiological conditions to give rise to stable tissue architecture. In contrast to canonical models of talin function, we define the essential role of the talin head to be as an orchestrator of integrin clustering and adhesion complex stability rather than as an integrin activator. We furthermore illustrate how specific inter-domain interactions in talin contribute to talin-dependent regulation of integrin function. Thus, our work sheds novel light on the molecular mechanisms that act through talin to underlie adhesion receptor clustering and adhesion complex stability, crucial aspects underlying tissue morphogenesis and homeostasis.
4.4 Materials and Methods

4.4.1 Molecular biology.

The generation of talinGFP is previously described [243]. 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 [243]. The making of pUASp-GFP-TalinHead was described previously [100]. This construct was directly mutated to contain the L334R point mutation using the QuikChange mutagenesis kit (Stratagene).

4.4.2 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\textsuperscript{79a} allele and the Dominant Female Sterile technique [254]. Females of the genotype \textit{yw, hs-Flp/+; pUbi-talinGFP, talinGFP*L334R, or headless-TalinGFP/+; rhea79a, FRT2A/OvoD1, FRT2A} were subjected to a heatshock-regime during the larval stages to generate mosaic germline in order to give rise to \textit{rhea} mutant oocytes with maternally supplied rescued transgenes. Virgins were then crossed to \textit{rhea}\textsuperscript{79a}/\textit{TM6b, dfd-GMR-nvYFP} males. Embryos without the fluorescent balancer were selected for analyses. Maternal-zygotic \textit{rhea}\textsuperscript{17} mutants were also generated via the Dominant Female Sterile germline-clone technique and crossed to \textit{rhea}\textsuperscript{17}/\textit{TM3, dfd-GMR-nvYFP} males. For all FRAP experiments, talinGFP constructs were heterozygous and expressed in a \textit{w}\textsuperscript{1118} background. UAS-driven transgenes were expressed in the muscle using
the muscle-specific mef2-Gal4 driver. For analysis of integrin clustering, 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, or rhea17, FRT2A/TM3, dfd-GMR-nvYFP. Larval progeny were subject to a heat-shock regime in order to induce clones and larvae in wandering third instar was selected for dissection and analysis.

4.4.3 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-αPS2-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; [239]). 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 a 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 1.0μm confocal sections. Embryos were staged as described in Ellis et al, 2013. For quantification of localization of IAC components to MTJs, all images were taken at the same settings. In all cases, fluorescence localization was measured using ImageJ and normalized to αPS2 levels. Statistical tests were performed using Prism5 software.
4.4.4 FRAP.

Stage 17 embryos were collected and prepared for FRAP as described previously [243]. 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 30% 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.
4.5 Chapter 4 Figures

(a) Schematic of key domains in talin. (b-c) Maternal zygotic talin null embryos rescued with either full-length WT talinGFP transgene (b) or talinGFP*L334R mutant transgene (c) and

Figure 4.1. Integrin-binding to the talin head, but not integrin activation, is required for muscle attachment.

(a) Schematic of key domains in talin. (b-c) Maternal zygotic talin null embryos rescued with either full-length WT talinGFP transgene (b) or talinGFP*L334R mutant transgene (c) and
stained for F-actin (green) and βPS-integrin (magenta). (d–f) Muscle attachments of talin null embryos rescued with either talinGFP-WT (d), talinGFP*R367A (e), talinGFP*L334R (f). Embryos were stained for anti-integrin (green in d–f; grey in d‘–f‘) and tiggrin, a Drosophila ECM molecule (red in d–f, grey in d“–f“). The R367A mutation, which prevents the interaction between the talin head and integrins, results in weakened attachment between integrins and the ECM (c; arrows). Surprisingly, specific disruption of talin-head dependent integrin activation using the L334R mutation (f) did not give rise to any defect in ECM attachment. (g) Activation of αIIbβ3 integrins by fly talin head constructs (WT or L334R) was measured in mammalian cell culture. The L334R mutation abrogates activation of human integrins. (h) Recruitment of ubi-promoter driven talinGFP and talinGFP*L334R to MTJs was assayed in wild-type embryos. TalinGFP*L334R competed less well with endogenous talin and was recruited less well to MTJs in comparison to WT talinGFP. (i) FRAP experiments on talinGFP-WT and talinGFP*L334R reveal that the mutant is less stable at sites of adhesion than WT.
Figure 4.2. The talin head is essential for integrin function in Drosophila. (a-c) Maternal-zygotic talin null embryos (shown in a) rescued with either full-length WT talinGFP transgene (b) or headless talinGFP transgene (c) and stained for F-actin (green) and integrin (magenta). (d-f) Integrin-dependent phenotypes dorsal closure (d), germband retraction (e) and muscle attachment (f) were assayed in talin-null embryos (yellow), WT-talin-rescued embryos (black), and headless-talin-rescued embryos (blue). The talin head was required for all three processes assayed. Scale bar=100 µm. (g) Recruitment of ubi-promoter...
driven, GFP-tagged full-length WT talin and headless talin to MTJs was assayed in wild-type embryos. Headless-talinGFP competed less well with endogenous talin and was only weakly recruited to MTJs compared to WT, which was robustly recruited. (h) FRAP experiments on talinGFP-WT and headless-talinGFP reveal that headless talin is much less stable at sites of adhesion than talinGFP-WT. (i-n) Confocal z-stacks of stage 17 maternal/zygotic-mutant embryos rescued with either full length WT talin (i,k,m) or headless-talin (j,l,n). (e-f) MTJs stained for talin (green in i-j; grey in i’-j’) and integrin (red in i-j). In the absence of the talin head, talin was still well recruited to MTJs. (k-l) Muscles stained for talin (magenta in k-l) and paxillin (green in k-l; grey in k’-l’). Paxillin was poorly recruited to muscle attachment sites. (m-n) Muscle attachments stained for zasp (green in m-n; grey in m’-n’) and filamin (to highlight muscle morphology; magenta in m-n). Zasp was not well recruited to muscle attachment sites.
Figure 4.3. *rhea*17 encodes a missense allele of talin which severely disrupts talin head function.

(a) The *rhea*17 allele is characterized by a missense mutation in a conserved glycine residue in the F3 lobe of the talin head FERM domain, G340E. (b-c) Whole mount stage 17 embryos stained for F-actin (magenta) and integrin (green) reveal that *rhea*17 mutant embryos (b) harbour severe morphogenetic phenotypes and muscle detachment defects compared to WT embryos (c). (d-f) WT and G340E talin head domains were overexpressed in muscle using the muscle-specific mef2-GAL4 driver. The G340E mutation impaired recruitment of the talin head to MTJs which was quantified by measuring the ratio of MTJ-localized talin head to cytoplasmic signal (f). (g) Activation of αIIbβ3 integrins by fly talin head constructs (WT or G340E) was measured in mammalian cell culture. The G340E mutation abrogates activation...
of human integrins. (h-l) WT and *rhea*¹⁷ embryonic muscles stained for talin (red in i-l; grey in i’-l’) and integrin (green in i-l) at stage 16 (i-j) and stage 17 (k-l). Talin was recruited well at all stages in *rhea*¹⁷ embryos indicating that the C-terminus of the protein, downstream of the mutation, is expressed and correctly folded. Recruitment of integrin to MTJs was also similar between WT and *rhea*¹⁷ embryos.
Figure 4.4. The G340E disrupts adhesion complex maturation and integrin clustering. 
(a-e) Muscles stained for PINCH (red in a-d; grey in a’-d’) and integrin (green in a-d) in stage 16 (a-b) and stage 17 (c-d) embryos. Localization of PINCH to MTJs was reduced compared to WT controls and was dramatically lost from late stage *rheas*17 embryos (e; normalized to integrin levels). (f-h) Stage 17 embryonic muscles stained for phospho-FAK (red in g-h; grey in g’-h’) and integrin (green in g-h). pFAK levels were significantly reduced in *rheas*17.
embryos compared to controls (f; normalized to integrin levels). (i-j) Clonal analysis reveals G340 is required to support talin-dependent integrin clustering in imaginal wing disc epithelia. Integrins form adhesive dot-like structures on the basal cell surface; they are not observed in mutant talin tissue (i) or in rheal mutant clones (j).

Figure 4.5. Proposed model for G340 function in maintaining F2-F3 domain organization.
(a-c) The conserved role of G340 (G331 in mammalian talin2) 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 (b). Modelling based on known structures (see Anthis et al, 2009 [64]) of talin 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, and interfering with integrin function (c).
CHAPTER FIVE: General Discussion and Conclusions

5.1 Overview of Findings

The goal of this thesis was to identify specific mechanisms of integrin regulation in the context of a developing, intact organism. I focused on talin, a core integrin adhesion complex component, and used a structure/function-based approach to confirm paradigms of integrin function established in cell culture systems. Importantly, we were able to extend on these findings in order to provide tissue-level insights into mechanisms of integrin function.

The major research conclusions of this work are as follows: (1) distinct interactions between an integrin heterodimer and its binding partner talin can promote diverse adhesive function in development; (2) talin activity must be precisely controlled in order to down-regulate integrin-mediated adhesion and facilitate cell motility during morphogenesis; (3) integrin activation-independent activities of the talin head are essential to promote integrin clustering via adhesion stabilization and reinforcement during tissue formation and maintenance.

Although descriptive roles for integrins have previously been identified in many different processes that occur during fly development, little headway has been made towards understanding the specific aspect of integrin function at play in each context. Using a unique structure-function approach, as well as combining live imaging, the powerful genetics of Drosophila, and immunofluorescence analyses, my work has defined physiologically relevant functions for various talin-dependent protein-protein interactions within an integrin adhesion complex. In addition to clarifying the role of integrin activation in Drosophila, the findings of
this thesis illustrate how a limited set of molecules within the integrin adhesome can ultimately dictate a broad suite of tissue behaviors by changing the way they interact with one another.

5.2 Emergent Themes and Implications of The Thesis

5.2.1 Tissue-specific mechanisms define distinct modes of integrin function

A common theme unifying this work is that organism-wide perturbation of specific aspects of integrin function usually resulted in phenotypes that manifest in a distinct subset of tissues that depend on integrin for their development. Broadly, the effects of the mutations we made can be divided into two categories: (1) those that disrupted morphogenetic movements and dependent on transient modes of integrin-mediated adhesion and (2) those that impinged on long-term adhesion in tissues where tissue architecture had already been stably established.

In Chapter Two, we describe a mutation, talinEGFP*R367A, which weakens the attachment between integrins and the ECM by disrupting IBS-1 binding to β-integrin cytoplasmic tails. This phenotype manifests itself only at the MTJs of the somatic muscles where integrins are required to stabilize tissue architecture following muscle morphogenesis. Interestingly, this mutation also gives rise to increased talin dynamics at sites of adhesion as revealed by FRAP experiments. Therefore, we postulate that when integrin-ECM attachment is weakened, the entire MTJ is destabilized leading to the observed muscle tearing. In Chapter Four, we also found that deletion of the talin head (headless-talinGFP) more than doubled the dynamics of talin at sites of integrin-mediated adhesion and severely disrupted maintenance of the muscle architecture. Based on these congruent results, we propose that the stability of talin at MTJs is essential for the maintenance of tissue architecture. Since MTJs in the embryonic
musculature persist for several days and throughout the course of larval life, their integrity is essential to the survival of the organism. Indeed, as the fly develops, IAC components at MTJs are known to down-regulate their dynamics to support increased forces imposed on them [244].

Interestingly, mutating IBS-1 had a negligible effect on morphogenesis; aside from the muscle-tearing defects, embryos appeared morphologically normal. This suggests that the success of highly dynamic tissue processes is not sensitive to changes that serve to increase adhesion complex dynamics. This is a somewhat surprising result, and there is likely a range of perturbation outside the upper limit of which morphogenesis would fail. In line with this prediction, we did observe some morphogenetic defects (albeit weaker ones than observed in complete absence of talin) when we abrogated talin head function. However, our analyses revealed functions for the talin head other than control of adhesion dynamics so the results are somewhat difficult to interpret.

The discovery that increasing the dynamics of integrin-mediated adhesions does not lead to failures in morphogenesis may help to reconcile the fact that many integrin-associated genes, such as vinculin, have been studied in Drosophila and seemingly do not give rise to embryonic phenotypes [242]. Loss of vinculin results in increased adhesion dynamics in the context of single cells migrating in 2-D [151]; this phenotype has consequences for the persistent directionality of a single migrating cell. However, in the context of a developing organism, there are presumably many partially redundant biochemical and biophysical signaling inputs that may be able to compensate and direct the process according to the developmentally programmed plan in spite of destabilized adhesions.
In contrast, we found that mutations that served to stabilize the integrin adhesion complex had deleterious effects on epithelial morphogenesis. For example, in Chapter Three, I describe how a mutation that constitutively forces talin into a more open, active conformation (talinGFP*E1777A) leads to a delay in DC, a process involving cell motility and rearrangement, as well as rapid cell shape change. Although we only delved into detailed analysis of DC, we also found that other morphogenetic processes were affected. For example, as reported in Figure 3.3, GBR was also impaired, and upon close examination of talinGFP*E1777A-rescued talin null embryos, I also observed mid-gut migration defects (data not shown). It is likely that further analysis would reveal failings in other epithelial tissues undergoing morphogenetic changes in the presence of this mutant talin molecule. Using FRAP analysis, we determined that the autoinhibition mutation rendered the talin molecule more stable at sites of adhesion, and furthermore, that talin and integrin localization was increased at sites of integrin mediated adhesion in the AS. This suggested that maintaining talin in an open conformation might lead to ectopic assembly of integrin adhesion complexes, thus leading to the observed delay in DC. Interestingly, we did not observe any defects in muscle attachment in talin autoinhibition mutant embryos. This finding lends itself to the idea that once tissue architecture is established, there is no such thing as too much integrin activity.

In Chapter Two, we found that mutating the second integrin binding site in talin, IBS-2, lead to defects in both DC and GBR. Moreover, we found that disrupting IBS-2 leads to stabilization of talin at adhesion complexes. The mechanism leading to this effect is more difficult to predict, but one possibility is that in the absence of IBS-2, talin can only interact with integrin through its N-terminal IBS-1 domain, which acts to stabilize cell-ECM attachment sites. Similar to what was observed for the talin autoinhibition mutants, this could
lead to hyper-stabilized adhesions, which cannot remodel quickly enough to facilitate changes in tissue architecture.

These results emphasize the importance of careful regulation of adhesion dynamics during morphogenesis. Both too much and too little adhesion can lead to defects. Indeed, this has been beautifully demonstrated in the extending germband of early *Drosophila* embryos, where differential regulation of the dynamics of various populations of cadherin-mediated adhesions underlies successful tissue remodeling and elongation [187]. My work has shed light on how the dynamics of integrin-mediated adhesions must also be regulated to give rise to tissue-specific modes of integrin function.

### 5.2.2 Talin as a scaffold for adhesion complex organization and behavior

While the field of cell biology has evolved past the one-molecule/one-function mindset for defining protein function, it continues to be rare to hear mention of talin without a closely followed reference to integrin activation. This thesis strongly argues that talin has many functions other than integrin activation, and in fact, provides evidence to suggest that talin-dependent integrin activation does not occur in *Drosophila*. Based on our data, we propose that scaffolding functions conferred by both the talin head and IBS-2, as well as linkage to the actin cytoskeleton prove to be the most important functions of talin in *Drosophila*.

My work supports the role of talin as a structural linker protein between integrins and the actin cytoskeleton. In the absence of talin, although integrin is recruited to sites of adhesion, other IAC components do not localize implicating talin as a master regulator of adhesion complex assembly. Our analysis reveals that, in large part, the talin head is responsible for this function of talin since recruitment of all IAC components we tested was
strongly reduced in talin null embryos rescued with headless-TalinGFP. Interestingly, we found that this function of the talin head might not depend on integrin binding since a mutation that prevented the binding of the talin head to integrin (talin*R367A) still efficiently recruited all adhesion complex components. Instead our studies suggested that the ability of the head to fulfill its scaffolding function might depend on a novel mechanism involving interaction between the F2 and F3 lobes of the talin head FERM domain. In this case, although linkage to the actin cytoskeleton via the C-terminal THATCH domain is not perturbed, muscle attachment is not maintained because talin head-dependent adhesion reinforcement is required to transmit force from the MTJ to the actin cytoskeleton. This closely mirrors what has been observed in cell culture, where talin is thought to play a key role in transmission of traction forces between the actomyosin network and focal adhesions [149, 150, 153].

Analysis of mutations that disrupt interactions of talin at its C-terminus also uncovered defects in IAC assembly and maintenance. For example, mutation of IBS-2 led to separation of IAC signal from integrin signal. Interestingly, in this case, the IAC remained associated with the actin cytoskeleton at muscle ends. This is similar to what was observed in rhea15-39 embryos; rhea15-39 encodes a nonsense allele of talin that contains a stop codon N-terminal to IBS-2, effectively deleting IBS-2 and the actin-binding THATCH domain [236, 246]. Both IBS-2 mutant embryos and rhea15-39 embryos display strong morphogenetic defects that are verging on dominant negative. Taken together, these results implicate a role for IBS-2 in maintenance of normal adhesion form and function, which is essential for morphogenesis. Exactly how IBS-2 may fulfill this role is unclear. One possible explanation is that binding between talin and integrin through IBS-2 frees the head to act as a scaffold and interact with binding partners such as FAK, Zasp, or Wech. If IBS-2 is mutated and talin can only bind to
Integrin through IBS-1 then this disturbs the usual balance of interactions the talin head makes leading to the observed defects in adhesion architecture.

Intriguingly, a mutation which blocks talin autoinhibition and presumably increases the ability of talin to act as a scaffold for adhesion complex assembly also leads to morphogenetic defects. However, we could not detect measurable changes in localization of IAC components to sites of adhesion other than that the integrin adhesion receptor itself. We postulate that this increase in membrane-localized integrins leads to too much adhesion in the amnioserosa. Nonetheless, it was still surprising that we did not observe changes in the localization of other adhesion complex components. Potentially this may be because the intramolecular interaction that mediates autoinhibition is in direct competition with the interaction between IBS-1 and β-integrin cytoplasmic tails rather than interactions between the talin head and any other binding partners [108].

An alternative explanation underlying the defects we observed is that blocking talin autoinhibition could promote increased linkage between talin and the actin cytoskeleton. Previous work from our laboratory revealed the importance of maintaining a direct THATCH domain-mediated attachment between talin and the actin cytoskeleton for DC [236]. How the ability of talin to autoinhibit might affect linkage to the actin cytoskeleton has not been well explored in vitro or in vivo. Possibly, if autoinhibition cannot occur, then talin may be more likely to bind to actin, thus limiting remodeling of the cortical cytoskeleton which would have deleterious consequences for cell shape change during dynamic morphogenetic defects. In line with this hypothesis, I observed gross disruption to the actin cytoskeleton in the leading edge epidermis during DC (data not shown), but we did not further explore this in the course of my analysis. Many mutations that impinge on actin remodeling have been identified to have
phenotypes in epithelial morphogenesis [212]. For example, Rap1, which we uncover as a regulator of talin autoinhibition during dorsal closure, is known to be required for mediating appropriate attachment between cell-cell adhesion complexes and the actin cytoskeleton through control of localization of the linker protein Canoe/Afadin. Loss of function phenotypes of Rap1 therefore lead to defects in mesoderm invagination and germband extension [274], processes that require constant remodeling of the actin cytoskeleton. Our analysis of both gain-of-function Rap1 transgenes, and transgenes that block Rap1 activity, revealed DC phenotypes upon their expression in the AS. Although we related our findings to regulation of talin localization and activity of integrin-mediated adhesions, it is likely that actin dynamics were also impaired. Therefore, efforts to understand the connection between Rap1 activity, talin autoinhibition and the actin cytoskeleton during dorsal closure would be an interesting area for future investigation.

The results of this study draw interesting parallels between regulation of talin activity and regulation of the activity of other FERM members. A two-step model for FERM protein activation has been proposed: (1) recruitment of FERM proteins to specific membrane domains (2) sequential release of the autoinhibitory interaction, first by membrane interactions, and second by phosphorylation, which becomes more energetically favourable following an initial unclasping event [128, 275]. For example, during cellularization of the Drosophila blastoderm, the synaptotagmin ortholog, Bitesize (Btsz), recruits the FERM protein moesin to the apical membrane where moesin is subsequently “activated” and acts to stabilize cadherins and the apical actin network [276]. Similarly, Ezrin is recruited to PIP2-rich membrane domains prior to activation [277]. Based on our data, we envision that a Rap1-dependent signaling pathway plays a similar role to specifically recruit inactive talin to
integrin-mediated adhesions, where it then can be activated and form a stabilizing link between integrins and the cytoskeleton.

5.3. Limitations and Proposed Future Directions

5.3.1 Reconciling differences between vertebrate and Drosophila integrins

One limitation of this thesis is that our findings may represent mechanisms of integrin regulation that are specific to *Drosophila*. For example, according to my studies, integrin activation is not a major aspect underlying talin-dependent control of integrin function in *Drosophila*. Whether this is truly a difference between *Drosophila* and other integrins or not remains to be seen. Importantly, the only population of mammalian cells in which an *in vivo*, physiologically relevant role for integrin activation has been described are blood cells, which act and travel through the blood stream as single cells rather than as part of a multi-cellular tissue structure [160]. In mice, platelet-specific knock-in of the Talin1*L325R* (equivalent to the fly talinGFP*L334R* mutant used in this thesis) demonstrated that talin-dependent integrin activation is specifically required for clot retraction and platelet adhesion. Although it has not been expressly demonstrated, it is also likely that abrogating talin-dependent integrin activation in a similar manner in leukocytes would have consequences for the ability of leukocytes to leave the vasculature since talin-dependent integrin activation has been implicated in this process [79, 161]. In our studies we did not examine the dispersal of hemocytes, the *Drosophila* blood cells, in talin mutant embryos rescued with the talinGFP*L334R* transgene, but it is possible that there may be defects in the tissue transmigration which regulates their even distribution across the embryo.
Our studies primarily focus on morphogenetically active epithelia (AS and epidermis) or multicellular tissues in which integrins maintain tissue architecture (muscle). Rather than talin-dependent control of integrin affinity, we found that talin dependent regulation of integrin clustering to be an essential mechanism through which talin contributes to integrin function. In vivo requirements for talin-dependent integrin activation or clustering have not been tested in mammalian epithelia or muscle, although clear roles for integrin and talin function have been demonstrated in these tissues via targeted loss of function studies [127, 166]. In a multicellular tissue, where integrin ligand density is high, it may be that integrins primarily regulate their affinity via outside-in signaling, and further fine-tune their function by receptor clustering. However, it remains to be elucidated whether this is the case in mammalian tissues, or whether this is a Drosophila-specific phenomenon.

Another possibility to explain our findings is that there is an evolutionary divergence in function between fly and mammalian integrins. It was reported that differences in the extracellular domains of fly and vertebrate integrins could underlie differential responses to talin head expression [99]. However, the basis of these differences was found to be technical: the assay used to measure integrin activation of mammalian integrins relied on the presence of multivalent ligands, whereas the Drosophila integrin activation assay used monovalent ligands. Correction of this technical discrepancy revealed the responses of mammalian integrins to talin head expression to be very similar to what was observed for Drosophila [95]. Furthermore, our own homology modeling (Ben Goult, unpublished observations) has not revealed any obvious differences in the way the talin head and integrin interact with each other compared to vertebrate integrins and talin.
5.3.2 How is integrin function regulated in the amnioserosa during dorsal closure?

The work presented in this thesis only begins to provide hints of how integrin function is regulated during DC. Nonetheless, it provides an important foundation from which to begin a systematic investigation of how integrin-mediated Cell-ECM adhesion is modulated and contributes to DC. I identified several mutations that impinge on dorsal closure including mutations in IBS-2 and mutations that block autoinhibition. For the autoinhibition mutant, we used live imaging to show that dorsal closure was delayed rather than fully disrupted. However, many questions remain with respect to how specific cellular functions are affected by this mutation, as well as by the IBS-2 mutation.

The contribution of mechanical forces to DC has become increasingly apparent during the last decade [214]. Since the efforts of my colleagues in our lab working on muscle development have identified the integrin adhesion complex as a mechanically-sensitive entity which changes its dynamics in response to acute changes in intracellular tension [244], it would be interesting to test how integrins contribute to biomechanical control of cell behavior during DC. Future work will use the various mutations in talin and integrin identified, by my work and the work of others, to have a DC phenotype to try to elucidate how cell shape change, actomyosin dynamics, and leading edge plasticity are altered in various mutants that disrupt DC.

An additional unanswered question with respect to the role of integrins in DC relates to the various tissue specific roles of integrin-mediated adhesion in DC. Three tissues participate in DC: the AS, the lateral epidermis, and the yolk. Integrin function has been implicated in all three tissues [225]; however, the exact roles of integrin in each tissue have not been delineated. In light of the fact that myosin re-expression only in the AS was sufficient to
rescue the DC phenotype of non-muscle myosin II (zipper) embryos [220], and that rescue of integrin function only in the AS conferred substantial rescue of GBR [211], another process that likely depends on mechanical contributions from the AS, we anticipate that basal cell-ECM adhesion will make a critical contribution to DC. It has been shown in prior analysis that integrin-mediated adhesion is required to maintain AS tissue integrity, but we further predict that basal cell-ECM adhesion will provide foci of traction force (perhaps mediated by FALS; see below), which facilitate contraction of the AS and ultimately lead to tissue closure.

During the course of my analysis, I identified a novel, integrin-containing structure on the basal surface of AS cells. Because of the close resemblance of these structures to focal adhesions, we term these structures Focal Adhesion-Like Structures (FALS). Work is ongoing to characterize the properties and dynamic regulation of FALS. We hypothesize that these structures are sites of cell-ECM adhesion between the basal amnioserosa and the underlying ECM layer. Experiments are underway to demonstrate that their formation is dependent on integrins. Our preliminary data shows that FALS contain talin and integrin and that they are associated with the actin cytoskeleton. We also have evidence to show that FALS are developmentally regulated: they change in size, shape, and dynamics throughout development, becoming less elongated, and more stable as dorsal closure proceeds. Interestingly, we observe a significant morphological change in FALS upon the application of compression forces. Based on this observation, combined with data showing that altering Rac1 or Rho1 activity has effects on FALS dynamics, we hypothesize that FALS are regulated by force. Finally, we have also identified particular mutations in talin and integrin that modulate the size, shape, and dynamics of FALS, suggesting that FALS function is controlled via specific integrin signaling mechanisms. This preliminary data is shown in Appendix A.
5.3.3 Understanding the role of too much adhesion during morphogenesis

In Chapter Three, we propose that the talin autoinhibition mutation leads to too much integrin-mediated adhesion and that this is the cause underlying the observed delay in DC. We base this hypothesis on two lines of evidence: (1) more talin and integrin are incorporated into integrin-mediated adhesions and (2) talin becomes more stable at sites of adhesion. My unpublished, preliminary work also demonstrates that FALS are enlarged in talin autoinhibition mutant embryos (see Appendix A). However, these are indirect measures of adhesive behavior. Just because we observe more integrin in adhesions, how do we know that the net cell-ECM adhesion per cell is in fact increased? In the context of a developing tissue, we cannot simply do an adhesion assay to measure how strongly AS cells are adhering to their substrate. Therefore, we are currently turning to quantitative biology and mathematical modeling approaches to address this question.

Individual cells within the AS undergo cycles of apical constriction and relaxation. As the cells change shape, we hypothesize that they move relative to one another to accommodate subtle and continuous changes in cell shape across a continuously constricting epithelium. We further hypothesize that the ability of cells to move in this context is dependent on the ability to remodel basal cell-ECM contacts, which presumably depends on integrins. We are currently taking high-time resolution time-lapse movies to track the centroid of cells in the AS to discern if there are differences in cell motility between wild type embryos and talin autoinhibition mutant embryos.

A cell-level biomechanical model of DC recently developed by our colleagues in the UBC Mathematics Department accounts for the friction factor that each cell within the AS is
subjected to during DC [221]. If friction is increased, then one might assume that cell mobility would be restricted. Since we hypothesize that the effect of disrupting talin autoinhibition is also to limit cell movement, we will use the model to test if increasing the friction factor also leads to a delay in the progression of DC. This will add further support to our claim that adhesion is increased by the talin autoinhibition mutation.

We can further validate our findings by testing the effect of other mutations that are known to increased integrin-mediated adhesion. For example, loss of function alleles of the kinase Msn are known to increase integrin surface expression and lead to impaired cell migration in the follicular epithelium [205]. Ablating Msn function during DC is therefore likely to lead to a similar effect on DC and AS cell motility as autoinhibition mutant talin.

5.3.4 Extension to mammalian systems and potential applications

Utilizing Drosophila as a model system has allowed us to delineate tissue-specific aspects of talin and integrin function in a relatively inexpensive and efficient manner. The major conclusions of this thesis give rise to hypotheses regarding how integrins are regulated in an intact multicellular organism. These hypotheses may now be tested in a mammalian system using tissue-specific knock-in approaches; my work provides some insight with respect to which mutations should be tested and in which tissue. For example, it would be of interest to test the effect of the talin-dependent integrin-activation mutant (talin*L325R) in muscle. Another interesting study may be to test the effect of the talin autoinhibition mutant (talin*E1770) in skin to see if there are defects in wound healing. The results of such efforts may have important implications for the design of therapeutics to treat pathologies that arise as a result of misregulated integrin function.
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APPENDIX A: Novel, Tension-Responsive Adhesive Structures Mediate Cell Attachment to the ECM during Dorsal Closure.

Appendix A Figures

Figure A1. Focal Adhesion Like Structures are found on the basal surface of the amnioserosa during dorsal closure.

(A) Confocal z-stack of the basal surface of the amnioserosa (AS) in a stage 14 embryo expressing talin-GFP (red) and stained for βPS-integrin (green). Outlines of the apical cell boundaries are traced in white. The leading edge is at the top of the image (thick white line),
anterior is to the left. (A’) shows a high magnification image of FALS. (A’’) Colocalization of Talin and βPS-integrin (yellow) in the amnioserosa assessed via Pearson Correlation scores (n=68 from 2 embryos). Negative control: Talin-GFP and Myosin Light Chain-Cherry (Squ- 

Cherry) do not colocalize at FALS. (B-C) FALS density and area was measured in the centre and the periphery of the AS. There is a significant increase in FALS density in the centre of the AS compared to the periphery, but no significant difference in size. (D) A subset of βPS-

positive FALS co-localize with F-actin. (E-G) FALS, labelled by anti-βPS (green in E,E’; white in F,F’), link to the basal actin mesh-work (labelled with phalloidin (magenta in E, E’; white in G,G’). Inset in E’-G’ shows a close up of boxed area in E. Note punctate colocalization between βPS and F-actin. (H) Time-lapse series of FALS labeled with talinGFP (green) and F-actin, labelled with Lifeact-Ruby (red). Arrowhead highlights a single FALS that is captured by a growing actin filament, and then tracks the tip. Bottom row shows talin-GFP channel in grayscale. Time is indicated as minutes:seconds, scale bar in E is 5 µm.
Figure A2. FALS are developmentally regulated. 

(A-B) Confocal z-stacks of talin-GFP labelling FALS in stage 12 embryos undergoing GBR (A) and stage 14 embryos undergoing DC (B). Note the distinct difference in FALS morphology between stage 12 and 14. Images shown for each stage correspond to the red-boxed area indicated on the cartoon schematics. The yellow arrows represent the position of the leading edge epidermis. Anterior is to the left. (C) FALS become more stable as dorsal closure proceeds as assessed by FRAP on talinGFP at specific developmental stages. Fluorescence recovery data were fit to one-phase exponential curves to determine the predicted mobile fraction of talinGFP at each developmental stage (C').
Figure A3. Evidence that FALS are sensitive to changes in both intracellular and externally applied forces.

(A-C) FRAP reveals the turnover of talin-GFP localized in FALS is upregulated when Rho function is disrupted by expression of Rho-DN in the AS (red; A,B) and downregulated when Rac function is disrupted by expression of Rac-DN in the AS (green; A,C). This data suggests that changes in actomyosin contractility might affect FALS behavior through a force-sensing mechanism (D-E) FALS visualized with talin-GFP in stage 14 embryos change morphology dependent on tissue compression in the presence or absence of a cover-slip bridge. Scale bar = 10µm.
Figure A4. Talin autoinhibition regulates FALS during dorsal closure.

(A-B) Single time-points from time-lapse movies of talin null embryos rescued with either talinGFP-WT (A) or talinGFP-E1777A (B; the E1777A mutation blocks talin autoinhibition). FALS in talinGFP-E1777A-rescued embryos are much more prominent than in talinGFP-WT rescued embryos, in which FALS are barely resolvable at this magnification. Dorsal closure is delayed in talinGFP-E1777A rescued embryos (see Ellis et al, 2013) suggesting that larger FALS could impair cell motility. Inset shows enlarged image of boxed area.