ELUCIDATING HOW PROTEIN TURNOVER IN CELL-ECM ADHESION STABILIZES TISSUE STRUCTURE DURING DEVELOPMENT

by

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B.Sc. Complutense University of Madrid, 2013

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(Cell and Developmental Biology)

UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

April 2016

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Abstract

Morphogenesis is the process by which cells rearrange to form complex three-dimensional structures. Cell to extracellular matrix (ECM) adhesion, primarily mediated by Integrins, is essential for the formation and maintenance of tissue architecture. A critical way to regulate cell-ECM adhesion is by modulating the turnover of Integrins and their adhesion complex, and thereby modulating the stability of Integrin-based adhesions. We previously showed that mechanical force stabilizes Integrin-based adhesions during development by modulating Integrin turnover. Here, we extend our studies to understand how mechanical stress impacts the dynamics of the cytoplasmic adaptor protein Talin, a critical regulator of Integrin function. Using Fluorescence Recovery After Photobleaching (FRAP) analysis in combination with a newly developed mathematical model that encompasses the complexities of Talin turnover, we determined that mechanical force stabilizes cell-ECM adhesion by increasing the rate of assembly of Talin-mediated adhesion complexes. To dissect the mechanisms that regulate Talin turnover downstream of mechanical force, we used point mutations of Talin which abrogate specific functions of the Integrin adhesion complex and measured turnover kinetics. We found that the activation of Integrins, resulting in increased affinity for ECM ligands, is a crucial process to regulate adhesion complex turnover. To further investigate the role of Integrin activation in regulating adhesion stability, we introduced small molecules known to induce “outside-in activation” of Integrins in vitro into live, intact embryos. This approach revealed that outside-in activation stabilizes cell-ECM adhesion by decreasing Integrin endocytosis; similarly to what we have previously seen when mechanical force is increased. Based on
this finding, we propose that mechanical force may induce changes in Integrin activation in order to stabilize cell-ECM adhesions. Overall, we show that Integrin activation is a key mechanism that regulates cell-ECM adhesion stabilization during embryogenesis.
Preface

Chapter 3.1: A version of this work has been published as:


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For this publication, Guðlaug Katrín Hákonardóttir carried the genetics and FRAP experiments for Figures 3.1, 3.2, 3.3, 3.4, 3.5 and 3.7. I performed the genetics and FRAP experiments for Figures 3.1, 3.6, 3.7, 3.10 and 3.11. Guðlaug Katrín Hákonardóttir and I made figures. Alejandra Donají Herrera Reyes, Raibatak Das and Daniel Coombs developed the mathematical model. Alejandra Donají Herrera Reyes performed the mathematical analysis. Guy Tanentzapf, Guðlaug Katrín Hákonardóttir and I conceived and designed experiments and analyzed data together. Guy Tanentzapf, Daniel Coombs, Alejandra Donají Herrera Reyes and I co-wrote the paper. Alejandra Donají Herrera Reyes and Daniel Coombs wrote the Supplemental materials and methods for the Talin mathematical model. In addition, Guðlaug Katrín Hákonardóttir published part of this work in her master’s thesis from the University of British Columbia.

Chapter 3.2 and 3.3:

I performed the all the genetics and FRAP experiments. Alejandra Donají Herrera Reyes and Daniel Coombs developed the mathematical model. Alejandra Donají Herrera Reyes performed the mathematical analysis. Guy Tanentzapf and I conceived and designed experiments and analyzed data together.

Appendix:

Alejandra Donají Herrera Reyes and Daniel Coombs wrote the Supplemental materials and methods for the Talin and Integrin mathematical model.
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<th>Description</th>
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<tbody>
<tr>
<td>ABS</td>
<td>Actin Binding Site</td>
</tr>
<tr>
<td>AMIDAS</td>
<td>Adjacent Metal-ion Dependent Adhesion Site</td>
</tr>
<tr>
<td>CLICS</td>
<td>Clathrin-Independent Carriers</td>
</tr>
<tr>
<td>DD</td>
<td>Dimerization Domain</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>e16</td>
<td>Embryonic stage 16</td>
</tr>
<tr>
<td>e17</td>
<td>Embryonic stage 17</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial Growth Factor</td>
</tr>
<tr>
<td>FA</td>
<td>Focal Adhesion</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal Adhesion Kinase</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence Recovery After Photobleaching</td>
</tr>
<tr>
<td>FERM</td>
<td>4.1 Protein Erzin Radixin Moesin</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphate</td>
</tr>
<tr>
<td>IAC</td>
<td>Intracellular Adhesion Complex</td>
</tr>
<tr>
<td>IBS1</td>
<td>Integrin Binding Site 1</td>
</tr>
<tr>
<td>IBS2</td>
<td>Integrin Binding Site 2</td>
</tr>
<tr>
<td>L1</td>
<td>First instar larvae</td>
</tr>
<tr>
<td>L3</td>
<td>Third instar larvae</td>
</tr>
<tr>
<td>LDV</td>
<td>Leu-Asp-Val</td>
</tr>
<tr>
<td>mef2</td>
<td><em>myocyte enhancing factor 2</em></td>
</tr>
<tr>
<td>MIDAS</td>
<td>Metal-ion Dependent Adhesion Site</td>
</tr>
<tr>
<td>MTJ</td>
<td>Myotendinous Junction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>mys</td>
<td>myospheroid</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-Buffered Saline</td>
</tr>
<tr>
<td>PINCH</td>
<td>Particularly Interesting New Cysteine and Histidine Rich Protein</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-biphosphate</td>
</tr>
<tr>
<td>PS</td>
<td>Position Specific</td>
</tr>
<tr>
<td>PSI</td>
<td>Plexin-Semaphorin-Integrin</td>
</tr>
<tr>
<td>PTB</td>
<td>Phospho-Tyrosine Binding</td>
</tr>
<tr>
<td>RGD</td>
<td>Arg-Gly-Asp</td>
</tr>
<tr>
<td>RIAM</td>
<td>Rap1-GTP-Interacting Adaptor Molecule</td>
</tr>
<tr>
<td>ROI</td>
<td>Region Of Interest</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SyMBS</td>
<td>Synergistic Metal Ion Binding Site</td>
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<tr>
<td>THATCH</td>
<td>Talin/ HIP1R/Sla2p Actin-Tethering C-terminal Homology</td>
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<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>uPAR</td>
<td>urokinase-type Plasminogen Activator Receptor</td>
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<tr>
<td>VBS</td>
<td>Vinculin Binding Site</td>
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<tr>
<td>WT</td>
<td>Wild-type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow Florescent Protein</td>
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Acknowledgements

First, I would like to express my gratitude to my supervisor Guy Tanetzapf and my committee members, Daniel Coombs and Don Moerman, for their scientific mentoring.

Next, I would like to thank my coworkers and collaborators, Guðlaug Katrín Hákonardóttir and Alejandra Donají Herrera Reyes for teaching me and executing this project with me.

Thank you so much crazy Tanentzoids for the fun moments: Katie (my first friend and blanket), Emily (classy), Michael (Mr. wonderful brain), Tina (don’t stop believing), Kaitlyn (is coffee ok?), Darius and Amanda (Ghostbusters), Katrín (the Icelandic Princess) Stephanie (playdoh time) and Fayeza (love). I will keep you all forever in my heart.

Theresa, thank you for believing in the colours of music and Lindsay, thank you for believing in magic.

I also feel very grateful for gecko and paleontology time with the Richman lab.

Thanks Azucena for always firing me up with Blondie and thank you my dark biologists for loving me as I am. Sara and Claudia weeeeee. All my love to my family.

Last but not least, thank you my lovely flies.
CHAPTER ONE: Introduction

1.1 Animal morphogenesis

Embryogenesis is the complex, generative, biological event that leads to the formation of a multicellular organism from a single cell. One of the most important aspects of embryogenesis is morphogenesis, the process by which cells rearrange in order to form highly organized and complex three dimensional structures. In combination with cell growth and differentiation, the tissue architecture achieved during morphogenesis gives rise to organs, which are responsible for the vital functions of the animal. This fascinating process drives the formation of multicellular organisms, and thus the study of developmental biology is of great importance to our understanding of life.

The question at the core of developmental biology is how do cells organize to form tissues and organs? Moreover, how do cells migrate, grow and achieve polarity to create a functional organ? To obtain such structural complexity, morphogenesis relies on morphogens, which are diffusive molecules that control cell fate by establishing concentration gradients along the body plan of the embryo. Once cell fate is generally determined, other cellular processes, such as cell division, shape change, migration, growth or death further the complexity and organization of various tissues. Some of these cellular responses are governed by the physical connection between cells (cell-cell adhesion) or between cells and the extracellular matrix or ECM (cell-ECM adhesion). Cell adhesion is
necessary in nearly every morphogenetic event, from embryogenesis to tissue homeostasis and the development of disease (Lecuit et al., 2011).

1.2 Cell adhesion and morphogenesis

1.2.1 Cell adhesion is required for morphogenesis.

Animal morphogenesis requires cell adhesion to assemble cells into tissues and to organize the in specific patterns. Cell adhesions connect structures outside of the cell to the cell interior and the cytoskeleton via a wide variety of adhesion complex components. This permits cell adhesion to transmit signals between the intra- and extracellular environments and to trigger genetic responses or effect changes in cell morphology or behaviour. Consequently, cell-ECM adhesions are able to translate cellular-generated genetic information into specifying where a cell belongs in the three-dimensional pattern of a tissue. The physical connections and the signalling conduits supplied by cell adhesion are an integral part of morphogenesis.

Adhesion complexes are generally composed of three types of proteins: a transmembrane adhesion receptor, ECM proteins and intracellular binding partners. Usually, adhesion is mediated by the extracellular domain of the adhesion receptor. The specificity of this domain determines the type of adhesion. In cell-cell adhesion the Cadherin family of proteins form different cells serve as both receptor and ligand, binding each other within the extracellular space with an IAC built within each respective cell. On the other hand, Integrins are the principal family of receptors that bind ECM ligands outside of the cell and build and IAC inside the cell, to mediate cell-ECM adhesion (Figure 1.1a).
Cell adhesions play diverse roles during morphogenesis. Two illustrative examples of the role of cell adhesion in morphogenesis are cell compaction and cell rearrangement. Cellular compaction consists in the connection of loose mesenchymal cells and their surrounding ECM to form a compact structure. In addition, tissues acquire diverse complex structures, such as tubes, branches or cysts by rearranging and changing the shape of cells. Integrins mediate both processes by regulating cell migration along the ECM.

1.2.2 The Integrin-mediated adhesion during morphogenesis.

Integrin-mediated cell-ECM adhesion is required for many developmental and homeostatic processes throughout the life of an animal. It is implicated in several cellular processes such as cell anchoring, migration, spreading and differentiation, which lead to important embryological events such as gastrulation, vasculogenesis and neurogenesis, (Bokel and Brown, 2002; Thiery, 2003). Thus, the role of Integrin has been and it is extensively studied in the field of developmental biology. For instance, mammals have several Integrin subunits and genetic removal of these subunits give rise to a variety of phenotypes, from embryonic lethality to wound healing defects (Sheppard, 2000). However, the study of mammalian Integrin biology is hindered by the redundancy of these many subunits. The model organism Drosophila melanogaster is a great system to study Integrins since fly Integrins are highly homologous to vertebrate Integrin, but are composed of combinations of far fewer subunits. Genetic removal of Integrins in Drosophila produces severe phenotypes in basic developmental processes such as germband retraction failure, dorsal closure failure, muscle detachment, and gut and tracheal defects that give rise to embryonic lethality (Brown et al., 1993). Also, the removal of the essential intracellular
binding partners of Integrin, for example the adaptor protein Talin, phenocopies the defects found in the Integrin knock outs (Bokel and Brown, 2002). Similarly, loss of ECM proteins (for instance Collagen and Laminin) can also lead to embryonic lethality (Brown et al., 1993).

1.3 Integrins, the key mediator of cell-ECM adhesion

1.3.1 Overview

As previously stated, the Integrin family of adhesion receptors is the main mediator of cell-ECM adhesion (Figure 1.1a). Integrins are heterodimeric transmembrane proteins composed of an α and a β subunit (Figure 1.1b) (Humphries et al., 2006; Hynes, 2002). In mammals there are 18 α subunits and 8 β subunits that dimerize in a non-covalent manner to form 24 different Integrin receptors (Humphries et al., 2006; Hynes, 2002). They have been highly characterized in Focal Adhesions (FA), which are in vitro protein-rich adhesion structures that transmit mechanical force from between the ECM and the actin cytoskeleton. *Drosophila* only has 5 α and 2 β subunits. *Drosophila* Integrins are historically known as Position-Specific (PS) given their differential expression pattern (Brown et al., 2000). They have 5 α subunits (αPS1-5) and two of the β subunits (βPS and βν) (Brown et al., 2000). The βPS subunit, encoded by the gene *myospheroid* (*mys*), is widely expressed and it dimerizes with all the α subunits, encoded by 5 different genes, to create 5 Integrin receptors (Brown et al., 1993). Moreover, it is well conserved with β1 and β3 in mammals. In contrast, the other β subunit, βν, is only expressed in endodermal cells of the gut. *Drosophila* is a very powerful model to study the roles of Integrins because of the reduced
number of possible Integrin heterodimers and therefore reduced redundancy compared to vertebrates (Yee and Hynes, 1993).

On the outside of the cell, Integrins bind a diverse array of ECM ligands, while on the inside of the cell they recruit a large variety of cytoplasmic binding partners which comprise the Intracellular Adhesion Complex (IAC) (Figure 1.1b). The IAC proteins allow for the diverse, yet precise functions of Integrin-mediated cell-ECM adhesions, and provide a structural connection to the cytoskeleton. For instance, Talin, Tensin and vinculin link Integrins to the actin cytoskeleton and transduce mechanical stimuli into cellular responses (del Rio et al., 2009; Delon and Brown, 2009; Liu et al., 2000). Integrin-Linked Kinase (ILK), Focal Adhesion Kinase (FAK) or Src kinases regulate cell-ECM function through protein phosphorylation (Geiger et al., 2009). Other important IAC components with specialized functions are Paxillin, Kindlin, PINCH, α-actinin and Welch (Geiger et al., 2009). Out of these many IAC proteins, the cytoplasmic adaptor protein Talin is the master regulator of Integrin function. Talin provides a direct structural link between Integrins and the actin cytoskeleton and acts as a mechanosensor in all cell-ECM adhesions (Figure 1.1b) (del Rio et al., 2009; Tadokoro et al., 2003).

1.3.2 Structural control of function

The general structure of Integrins is evolutionarily conserved across species. Each Integrin subunit has an extracellular domain, a Transmembrane Domain (TM) and a short cytoplasmic tail (Figure 1.2) (Arnaout et al., 2005; Xiao et al., 2004). The conformation of these domains and their association with the Integrin binding partners determine Integrin function. Integrins are known to have several conformations that regulate their affinity to
the ECM ligands: a bent (low affinity), an intermediate, and an extended (high affinity) conformation (Figure 1.2) (Luo and Springer, 2006). Integrin activation is the process by which bent Integrins undergo a conformational change that extends their extracellular domains and separates the cytoplasmic tails by dissolving the salt bridge in the cytoplasmic domain in order to gain affinity to the ECM ligands (Anthis et al., 2009). Despite the variability of many Integrin subunits across different species, Integrin activation is thought to be a conserved process

1.3.3 Extracellular domain of Integrin

The structure of the Integrin α and β subunits has been extensively studied in vitro. They are formed by approximately 1000 and 750 amino acids respectively. The length of the extracellular domain depends on the conformation of the molecule: ~11nm in the bent conformation transitioning to ~19-23nm in the active conformation. The extracellular domain is responsible for the interaction with ECM proteins. The α subunit extracellular domain is composed of 4 to 5 different subdomains: αI, β-propeller, thigh domain and two calf domains, from the distal end to the cell membrane (Figure 1.2) (Campbell and Humphries, 2011; Shattil et al., 2010). The αI domain is only present in 9 of the 18 mammalian α subunits (Campbell and Humphries, 2011; Larson et al., 1989).

The β subunit extracellular domain is formed by 8 subdomains: βI, hybrid, Plexin-Semaphorin-Integrin (PSI), four Epithermal Grow Factor (EGF) and β-tail domains, from the distal end to the cell membrane (Figure 1.2) (Campbell and Humphries, 2011; Humphries et al., 2004; Shattil et al., 2010). The βI in the β subunit is homologous to the αI domain of the α subunit (Campbell and Humphries, 2011). ECM ligand binding depends on
cation binding to the Integrin receptor. The βI domain contains 3 cation binding sites. Mg\(^{2+}\) interacts with the Metal-Ion Dependent Adhesion Site (MIDAS) and this is required for ECM binding (Zhu et al., 2008). The MIDAS site is flanked by the Synergistic Metal ion Binding Site (SyMBS) and the Adjacent Metal-Ion Dependent Adhesion Site (AMIDAS) (Campbell and Humphries, 2011). Both of these flanking sites are the target of the inhibitory ion Ca\(^{2+}\) (Humphries et al., 2003; Zhu et al., 2008). Mn\(^{2+}\) replaces Ca\(^{2+}\) at the AMIDAS site to induce Integrin activation (Humphries et al., 2003). Though this has been extensively studied in β1, it has been suggested that the αI domain also contains a Mg\(^{2+}\) dependent MIDAS site (Lee et al., 1995b). Hence, divalent cations are able to regulate ligand binding affinity and stimulate conformational changes in Integrins (Gailit and Ruoslahti, 1988; Mould et al., 1995).

In addition to divalent cation binding, movements of the α helices of the I domain of both α and β subunits are also implicated in activation. Specifically, in αI-less Integrins, the βI domain has open and closed conformations based on the movement of the 7α helix (Luo et al., 2007; Xiao et al., 2004); therefore, the movement of 7α helices can contribute to activation (Lee et al., 1995a). Moreover, similar behaviours have been observed in the αI domains (Campbell and Humphries, 2011).

ECM ligand binding occurs at the interface between the β-propeller and the βI domain in αI-less Integrins. A large set of ECM proteins are able to bind to Integrins because they share common acidic peptide motifs (Campbell and Humphries, 2011). ECM ligands can be classified based on these common peptides. One of the most representative examples is the Arg-Gly-Asp (RGD) motif, contained in important ECM components such as Collagen and Laminin. ECM proteins containing this motif bind at the interface between
the β-propeller and the βI domain in αI-less Integrins due to the interaction between the Mg$^{2+}$ located in the MIDAS site with the Asp of the ligand (Campbell and Humphries, 2011; Shattil et al., 2010). Another example is the Leu-Asp-Val (LDV) motif, which bind at the interface of the α and β subunits in a similar way as RGD peptides (Campbell and Humphries, 2011).

1.3.4 Transmembrane domain of Integrin

The transmembrane domain of each Integrin subunit is composed of a ~4nm, single membrane spanning helix (Campbell and Humphries, 2011). It is known that the α and β subunits interact at this point to hold an inactive conformation (Campbell and Humphries, 2011; Wegener and Campbell, 2008). Nuclear Magnetic Resonance (NMR) studies of the αIIbβ3 Integrin show that the αIIb subunit has a perpendicular orientation with respect to the cell membrane (Lau et al., 2009), while the β3 subunit is inclined (Lau et al., 2009).

1.3.5 Cytoplasmic tail of Integrin

Integrins have an ~8nm flexible cytoplasmic tail that can bind to IAC proteins (Legate and Fassler, 2009; Shoemaker et al., 2000; Wegener and Campbell, 2008). The β subunit tail has the most important protein-protein binding sites. It has a membrane proximal and a membrane distal NPxY motif where Talin and Kindlin bind, respectively (Wegener and Campbell, 2008). This makes the β subunit cytoplasmic tail a major component of IAC signalling events; these will be explored further in this thesis. Additionally, the salt bridge that connects both Integrin subunits is located in the
cytoplasmic tail (Campbell and Humphries, 2011; Shattil et al., 2010). The disruption of this bridge is necessary to complete Integrin activation as stated above (Anthis et al., 2009).

1.3.6 Integrin signalling: Integrin activation

Aside from structurally linking the ECM to the inside of the cell, Integrins have an important role in cell signalling. One of the main ways Integrin signaling is achieved is by changing the conformation of Integrins (Integrin activation) and in turn, its affinity to the ECM ligands. Integrin activation is a bi-directional process that triggers several signalling events that give rise to cellular responses such as adhesion maturation. Activation occurs in two directions: from the inside (inside-out activation) and from the outside (outside-in activation). Despite of being caused by different sources, in vitro studies have shown that Talin binding to the cytoplasmic tail of the Integrin β subunit is required in both cases of activation (Tadokoro et al., 2003).

1.3.7 Inside out Integrin activation

Inside-out activation is the process by which Integrins gain affinity to the ECM ligands due to an intracellular signal communicated by IAC molecules such as Talin or Kindlin. Inside-out activation is promoted by the binding of phospho-tyrosine binding (PTB) domains of Talin and Kindlin to the NPxY motifs of the Integrin β subunit.

Talin is a large cytoplasmic protein (approximately 2450 amino acids) composed of an N-terminal FERM (4.1 Protein Erzin Radixin Moesin) head that contains four subdomains (F0-3) and a large C-terminal rod composed of 14 subdomains (R1-13 and DD) (Critchley, 2009). In vitro work has suggested that only the head domain of Talin
promotes Integrin activation (Calderwood et al., 1999). To activate Integrin, the F3 PTB domain of the Talin head binds to the membrane proximal NPxY motif of the β Integrin (Calderwood et al., 2003; Calderwood et al., 2002). Binding of the F3 domain causes disruption of the salt bridge in the cytoplasmic domain of Integrin due to competition between a lysine of Talin and an aspartic acid of α Integrin, and in turn promotes separation of the α and β subunits (Anthis et al., 2009; Wegener et al., 2007). Moreover, the F2 domain of Talin repositions the β subunit to achieve spatial separation between α and β Integrins (Anthis et al., 2009; Critchley, 2009; Kim et al., 2003). It has been also suggested that the orientation of F2 with respect to cell membrane creates a membrane orientation patch that has a crucial role in Integrin activation; furthermore, the residues responsible for the creation of the positively charged patch are evolutionary conserved (Anthis et al., 2009). Ultimately, the disruption of the salt bridge and the binding of the F2 domain results in a 20° inclination in the β subunit that leads to Integrin activation and thus increased Integrin affinity for ECM ligands (Anthis et al., 2009).

Kindlin is another member of the IAC. Similarly to Talin, the Kindling F3 PTB within its FERM domain binds to the NPxY motif of the Integrin β subunit (Harburger et al., 2009; Moser et al., 2008). However, Kindlin binding on its own is not sufficient to promote Integrin activation and it is considered a co-activator of Integrins with Talin (Ma et al., 2008).

1.3.8 Outside in Integrin activation

Outside-in activation is the process by which Integrin binding to the ECM components elicits an intracellular signalling response (Humphries et al., 2006). ECM
binding controls the extension, and thus activation, of the Integrin receptor though divalent cation binding as previously described in this thesis (Section 1.3.3). In response to ECM ligand binding, Integrins undergo a conformational change to gain affinity to the ECM. The conformational change also affects the cytoplasmic tail, which triggers signalling events inside the cell.

One of the most important signalling responses related to outside-in activation is FAK signalling. FAK is an IAC scaffold protein composed of a N-terminal FERM-domain that interacts with the plasma membrane and a C-terminal domain that connects to other IAC components (Deakin and Turner, 2008). Due to this physical interaction with the membrane and the IAC (and in turn the actin cytoskeleton), FAK activity is conditioned by mechanical force (Hytonen and Wehrle-Haller, 2015). FAK is a major regulator of adhesion dynamics, and acts by promoting IAC protein recruitment and F-actin cytoskeleton rearrangement.

1.3.9 Integrins and mechanical force transmission

Mechanotransduction is the translation of mechanical forces into a cellular biochemical response. Multiple adhesion receptors are implicated in force transmission, including Integrins and Cadherins. Integrins experience mechanical stimuli produced by cellular contraction or extrinsic factors (occurring in phenomena like shear stress, osmotic forces, morphogenetic forces, etc), which leads to FA maturation. FA maturation in response to mechanical force can occur through conformational changes of IAC proteins, such as Talin. Importantly, Integrins themselves are unable to produce force and therefore transmit it (Schwartz and DeSimone, 2008). Morphogenesis is driven by numerous
mechanical forces, which are in turn transmitted via cell-cell and cell-ECM adhesion. Thus, scientists are very interested in elucidating the mechanisms by which mechanical force regulates cell-ECM adhesion in order to successfully complete morphogenesis.

1.4 Talin, the main regulator of cell-ECM adhesion

1.4.1 Overview

As mentioned before, Talin is a large cytoplasmic protein composed of an N-terminal FERM domain-containing head and a large, flexible C-terminal rod (Figure 1.3) (Critchley, 2009). Vertebrates have two genes that encode for Talin, *tln1* and *tln2*. Despite being very similar (74%), Talin1 is the most important of the two. Significantly, flies only have one Talin, which is conserved to the mammalian Talin1.

The structure of mammalian Talin1 has been extensively characterized, and shown to be extremely similar to *Drosophila* Talin. The Talin FERM head is composed of four subunits, F0, F1, F2 and F3. The C-terminal rod is formed by 61 α helices that associate into 13 helical bundles (R1-R13, each bundle contains 4 or 5 helices) plus a single α helix that forms the Dimerization Domain (DD) (Goult et al., 2013). The head and the rod are connected by an unstructured linker region (residue 401-481). This linker region is rich in phosphorylation sites (Ratnikov et al., 2005) and it can be cleaved by certain proteases (Rees et al., 1990) in order to separate the head form the tail. Talin orchestrates Integrin-based adhesions by recruiting IAC components though its multiple protein-protein binding domains along the head and rod.
1.4.2 Talin binding partners and domains

The Talin head and rod domains contain many binding sites crucial for the regulation of both Integrin and Talin function. The Integrin Binding Site 1 (IBS1) is located in the F3 domain, and binds to the membrane proximal NPxY motif of the Integrin β cytoplasmic tail to trigger Integrin activation (Calderwood et al., 2002). At the F2 and F3 domains, there is an Actin Binding Site (ABS) (Lee et al., 2004). The F3 domain is also able to bind to several other proteins including FAK, Layilin and PIP-kinases (Calderwood et al., 2013; Critchley, 2009). The second Integrin Binding Site (IBS2) is located at the rod (R11-R12) (Rodius et al., 2008). Talin can bind Integrin at this domain, but this binding does not promote Integrin activation (Rodius et al., 2008). Work in Drosophila has confirmed that Talin triggers Integrin activation and increased ECM ligand binding affinity thorough IBS1 (Ellis et al., 2011). In contrast, IBS2 only helps stabilizing the connection between Integrins to the IAC (Ellis et al., 2011). Furthermore, there are two ABS at the rod (R4-R8 and R13-DD) (Hemmings et al., 1996). The C-terminal Talin/HIP1R/Sla2p Actin-Tethering C-terminal Homology (THATCH) domain is well characterized: the single α helix at the DD contributes to Talin dimerization and the 5 α helices of the R13 domains create a pocket where actin binds (Critchley, 2009). Also, Talin activity is auto-regulated by the binding of the F3 domain in the head to the R9 bundle in the rod (this is reviewed in further detail in section 1.4.3). Finally, there are several Vinculin Binding Sites (VBS) throughout the rod domain (Gingras et al., 2005).
1.4.3 Talin activation and recruitment

Talin recruitment to adhesions follows several pathways which terminate in Talin-Integrin binding. These pathways all depend on the ability of Talin to regulate itself. Talin activity is auto-regulated by the binding of the F3 domain in the head to the R9 bundle in the rod, creating a closed and inactive conformation termed autoinhibition (Anthis et al., 2009; Calderwood et al., 2013). Once Talin adopts this conformation, IBS1 in F3 is masked as it this site overlaps with Talin rod binding. Recruitment of autoinhibited Talin is mediated by the small GTPase Rap1 (Ras-proximate-1 or Ras-related protein 1) in combination with Rap1-GTP-Interacting Adaptor Molecule (RIAM) (Bos, 2005). Rap1 recruitment of Talin to the IAC promotes its activation which in turn promotes Integrin activation. Rap1-dependent recruitment occurs through RIAM binding to RIAM binding sites within Talin. Alternatively, Phosphatidylinositol 4,5-biphosphate (PIP2) also modulates Talin auto-inhibition by alleviating the interaction between F3 and R9 (Critchley, 2009).

FAK is also an important regulator of Talin recruitment. It has been suggested that Talin can be recruited to the membrane in an Integrin-independent manner via FAK. Furthermore, Talin is not present in early FAs in absence of FAK (Calderwood et al., 2013).

1.4.4 Talin as a mechanosensor

Talin is central to the IAC functioning in mechanotransduction. Talin acts as a mechanosensor by undergoing conformation changes in response to increased mechanical force. This conformational change exposes cryptic protein-protein binding sites within Talin, which leads to changes in signalling events at the IAC (del Rio et al., 2009).
Mechanical stimuli come from the outside of the cell through Integrins of from the inside through actin-dependent contraction (Margadant et al., 2011) An example of changes in signalling that occur via Talin in response to force include the exposure of vinculin binding sites. Since VBS are now exposed, more vinculin is recruited to the IAC, making it more stable. It has also been suggested that force acts on the first helix of the R13 domain, giving rise to high affinity binding of actin to the R13 bundle (Calderwood et al., 2013; Gingras et al., 2008).

1.5 The ECM

1.5.1 ECM composition

The ECM is a network of diverse proteins secreted by cells to the extracellular environment. The major functions of the ECM are: structural support and anchorage for cells through cell-ECM adhesion, tissue limit definition and cellular communication assistance. In animals, the ECM can be arranged loosely into an interstitial web or tightly into a basement membrane, a well-defined sheath of ECM that surrounds certain tissues. The ECM is composed of diverse families of proteins, including fibrous proteins, glycoproteins, proteoglycans and glycosaminoglycans (Broadie et al., 2011). These proteins are synthetized inside the cell, then secreted by exocytosis to the extracellular environment where they aggregate to form an interlocked mesh (Reichardt and Prokop, 2011) and bind to several adhesion receptors (e.g. Integrin, Syndecans and the Dystrophin-associated glycoprotein complex) (Bokel and Brown, 2002; Waite et al., 2009). The most common examples of ECM proteins are Collagen, Elastin, Laminin, Perlecan and Fibronectin. Collagen is the most abundant protein in the ECM and, in fact, the most abundant protein in
the human body. It forms resistant fibres which provide structural support. In contrast, Elastin fibres provide elasticity to the tissues. Laminin is a cross-linked that forms basement membranes. *Drosophila melanogaster* is a great model to study ECM since ECM proteins and their adhesion receptors are evolutionary conserved. *Drosophila* ECM includes two Laminins: Laminin A, whose subunits are encoded by the genes *lanA*, *lanB1* and *lanB2* and a minor Laminin W, as well as type IV Collagen, encoded by the genes *cg25C* and *vkg* (Broadie et al., 2011).

**1.5.2 The ECM also drives morphogenesis**

The ECM is vital to complete morphogenesis, as it serves as the substrate for cellular attachment, migration and polarity. Moreover, ECM localization and turnover can determine the growth pattern of a developing structure (Gumbiner, 1992). In the case of *Drosophila* development, ECM proteins are mainly secreted by the fat body (the homologous of a liver), and haemocytes (immune cells). Furthermore, ECM proteins are synthetized and deposited after 5-10 hours in development, in fact, the ECM has been completely deposited along the body by the last embryonic stage (e17) (Fessler and Fessler, 1989).

**1.6 The turnover of the Integrin-mediated adhesion**

**1.6.1 Overview**

Between 1980 and 1990s, Mark Bretscher discovered that Integrins undergo cycles of exocytosis and endocytosis to and from the plasma membrane (Bretscher, 1989; Bretscher, 1992; Bretscher and Aguado-Velasco, 1998). Since the simplest way to regulate
cell-ECM adhesions is to control Integrin availability on the cell membrane, this endocytosis-dependent turnover was proposed to be a core mechanism for determining adhesion stability.

Advances in fluorescence microscopy methods have allowed us to further dissect the kinetics of protein turnover. For example, FRAP of fluorescently-tagged proteins is a common and reliable technique used to analyze turnover. FRAP measures the recovery of a fluorescently tagged protein over time after a photobleaching event (Figure 1.4b, d) (Reits and Neefjes, 2001). From the recovery of fluorescence intensity, it is possible to calculate the mobile fraction of the protein, i.e. the proportion of proteins undergoing turnover. The mobile fraction is defined as the ratio of mobile proteins to the total amount of proteins (Figure 1.4d) (Reits and Neefjes, 2001). In the case of adhesions, a high mobile fraction indicates low adhesion stability. In comparison, a low mobile fraction means high stability.

1.6.2 Integrin trafficking in cell culture

The first step in the Integrin traffic route is the synthesis of the α and β subunits in the endoplasmic reticulum, and proper assembly and folding (Huang and Springer, 1997; Lu et al., 1998). Integrins are prevented from being sent to the cell membrane unless they acquire their native conformation (Huang and Springer, 1997). Once at the membrane, Integrins undergo constant recycling cycles (Bridgewater et al., 2012). A pool of Integrins can be completely removed from the plasma membrane in approximately 30 minutes, and the half-life of Integrins at the membrane is 12 to 24 hours (Paul et al., 2015).

Integrin internalization by endocytosis has been well characterized. It is known that inactive and active Integrins undergo endocytosis (Paul et al., 2015). Integrins can be
endocytosed in a Clathrin dependent manner. The Clathrin adaptors Dab2, Eps 8 and Numb mediate Integrin endocytosis through their PTB domains by directly binding to the NPxY motif in the cytoplasmic β tail (Calderwood et al., 2003). Also, the GTPase Dynamin-2, recruited by FAK, contributes to Clathrin-dependent Integrin endocytosis by closing the neck of the forming vesicle (Ezratty et al., 2005). Integrins can also be endocytosed via a Clathrin-independent pathway through Clathrin-Independent Carriers (CLICS) and caveolae (Bridgewater et al., 2012).

Once Integrins are internalized, they are directed to early endosomes where they can be sent for degradation in late endosomes or recycled back to the membrane (Caswell and Norman, 2006). Integrin recycling is mediated by the Rab and Arf GTPase families (Bridgewater et al., 2012; Paul et al., 2015). Integrins in early endosomes can be quickly returned to the membrane through Rab4 (di Blasio et al., 2010; Jones et al., 2009; Roberts et al., 2001) or they can follow a longer pathway across the perinuclear recycling compartment through Rab11 and Arf6 (Caswell and Norman, 2006; Powelka et al., 2004; Roberts et al., 2004).

Endocytosis-mediated Integrin turnover is required in cell migration in culture models; Integrins that anchor the rear of the cell to the substrate must be endocytosed and recycled to the front of the cell to allow the cell to move forward (Bretscher and Aguado-Velasco, 1998). Recently, it has been demonstrated that Integrin endocytosis is also responsible for trafficking Integrins between different regions at the front of the cell, and between the perinuclear area and the cell rear (Paul et al., 2015).
1.6.3 In vivo turnover analysis of the Integrin-based adhesion components

Despite the importance of turnover in cell-ECM adhesion regulation, this phenomenon has been poorly characterized in vivo. FRAP analysis in Drosophila Myotendinous Junctions (MTJS) (Figure 1.4c) has shown that Integrin and several IAC components such as Talin, ILK and Tensin undergo turnover (Yuan et al., 2010). Furthermore, this phenomenon is dependent on Clathrin dependent endocytosis and Rab5 recycling (Bottcher et al., 2012). In addition, cell-ECM adhesion becomes more stable over the course of fly development as evidenced by a reduction in the mobile fraction of Integrin-based adhesion components at subsequent embryonic and larval stages (Pines et al., 2012; Yuan et al., 2010). Aside from the already mentioned molecular mediators of adhesion turnover, in vivo FRAP analysis in combination with mathematical modelling has demonstrated that mechanical force stabilizes cell-ECM adhesion by regulating Integrin turnover, and specifically by decreasing the endocytosis rate of Integrin (Pines et al., 2012).

1.7 Aims, hypothesis and rationale

The main aim of my research is to determine how Integrin-mediated adhesion is regulated to accomplish morphogenesis and to ensure long term maintenance of tissues. Specifically, I focus on the regulatory mechanisms that control the turnover of Integrin-mediated adhesion components and in turn, how turnover controls the transition between morphogenesis to tissue maintenance.

Aim 1: To understand the effect the effect of mechanical force on Talin turnover and to aid in the development of a mathematical model that describes turnover of Talin. The primary goal of this aim is to provide mechanistic insight into how Talin regulates the
stability of Integrin-mediated adhesion in response to the application of mechanical force. My hypothesis for this aim is that Talin is able to regulate the turnover of the IAC leading to the stabilization of the adhesions in response to force.

Aim 2: To determine the effect of outside-in activation on Integrin turnover and how this is coupled to the regulation of Integrin turnover in response to mechanical force. We hypothesize that Integrin outside-in activation places a definitive role in cell-ECM stabilization by regulating Integrin turnover.

Aim 3: To investigate ECM turnover in vivo. Our goal is to shed light into how ECM turnover is also a key regulator of animal morphogenesis. Since two of the major components of the Integrin-mediated adhesion undergo turnover, the Integrin receptor and the IAC proteins, we believe that the third major component, the ECM, also undergoes turnover in vivo.

The Tanentzapf lab has shown that the Integrin-based adhesion components undergo turnover (Yuan et al., 2010). Moreover, we have demonstrated that mechanical force regulates turnover of Integrins and some molecules of the IAC and consequently, that force transmission through cell-ECM adhesion is essential for the transition between morphogenesis and long-term tissue maintenance (Pines et al., 2012). In our model organism, Drosophila melanogaster, we use FRAP in embryonic and larval MTJs in order to quantify turnover (Figure 1.4) (Schweitzer et al., 2010). MTJs are the place where epidermal-tendon and muscle attach through cell-ECM adhesion (Schweitzer et al., 2010). MTJs are great models to study turnover because they are rich on the Integrin-based adhesions, and close to the transparent epidermis. Together, these features allows us to
perform FRAP analysis in an intact alive organism. Furthermore, *Drosophila* is an ideal system for our research because it allows us to apply physiologically relevant mechanical force due to muscle contraction *in vivo* conditions. Tensile force can be varied at MTJs using temperature sensitive mutants that alter the degree of muscular contraction (Pines et al., 2012). Together, these results support that cell-ECM adhesion turnover as a key regulator of morphogenesis. Moreover, it is also possible to chemically manipulate cell-ECM adhesion by introducing small molecules into embryos *in vivo*.

My research will addresses fundamental biological questions regarding the regulation of cell adhesion, which is applicable to many crucial developmental processes. Furthermore, despite being one of the most important regulatory mechanisms of cell-ECM adhesion, turnover has not been extensively studied *in vivo*. 
1.8 Chapter 1 figures

Figure 1.1. Main types of cell adhesion: cell-cell and cell-ECM adhesion. (a) Cell to cell adhesion is mainly mediated by the Cadherin family of adhesion receptors (green) while cell-ECM adhesion is mediated by the Integrin family of adhesion receptors (blue and orange). (b) Schematic of the Integrin adhesion complex.

Figure 1.2. Schematic of Integrin conformational change and structure. Integrins are heterodimeric transmembrane proteins composed of an α (orange) and a β (blue) subunit. Each subunit has an extracellular domain, transmembrane domain and a cytoplasmic tail. αI-less Integrins lack the αI domain while αI Integrins contain an αI domain in the alpha subunit. The cation binding sites (MIDAS, AMIDAS, SyMBS) are located at the β I domain of the beta subunit. This schematic shows the bent conformation of Integrin (low affinity to the ECM ligands) and the extended or active conformation (high affinity to the ECM ligands).
Figure 1.3. Schematic of Talin structure and binding domains. Talin is composed of a FERM head (domains: F0-3) and a rod (domains: R1-13 and DD) which are connected by a linker region. In the Talin head some of the most important binding domains are: ABS, IBS1 and FAK domain. In the rod some relevant domains are: two ABS, three RIAM, IBS2 and multiple VBS (pink circles).

Figure 1.4. FRAP approach to analyze cell-ECM adhesion turnover in Drosophila MTJs. (a) Embryonic (stage 16) Drosophila MTJs visualized using Talin-GFP. (b) Example of a FRAP experiment. Fluorescence is measured (orange rectangle) after a photobleaching event (red circle) (c) Schematic of a Drosophila MTJ. MTJs are rich in the Integrin adhesion complex and close to the epidermis, which is transparent, making them an excellent model to study cell-ECM adhesion turnover. (d) Typical FRAP curve (fluorescence recovery) and mobile fraction of a protein. (e) Mathematical model that describes Integrin turnover using two parameters: the endocytosis rate ($k_{\text{endo}}$) and the exocytosis rate ($k_{\text{exo}}$) of Integrin. Scale bar: 20μm.
CHAPTER TWO: Materials and methods

2.1 Fly genetics

For FRAP analysis, we visualized Talin in vivo using Talin flies with a single copy of the wild-type or point mutated pUBI-talin-GFP transgenes in a wild-type background (Yuan et al., 2010). The talin mutants used were as follows: K2450D/V2451D/K245D (Franco-Cea et al., 2010), K2094D/S2098D (Ellis et al., 2011), R367A (Tanentzapf and Brown, 2006), and L334R (Ellis et al., 2014). Brkd129/TM3 and paraa2 were provided by J. Troy Littleton (MIT, Cambridge, MA) and are described elsewhere (Montana and Littleton, 2004). Fluorescent talin transgenes in either a Brkd129 (embryonic stage 17) or paraa2 (in males (as paraa2 is on the X) of third instar larvae, since the contractility phenotype manifests at this stage) heterozygous background were used to perform FRAP analysis. To change FAK activity, we used an inactive version of FAK (UAS-FAK-Y430F) expressed specifically in the muscle using the muscle driver mef2GAL4 in combination with WT-talin-GFP. For the Talin developmental series, a copy of pUBI-talin-GFP was expressed in a rhea79 heterozygous null mutant background. Embryos with a copy of pUBI-talin-GFP were used in endocytosis inhibition experiments.

In studies of Integrin turnover, we visualised Integrin using flies that had a single copy of the wild-type or point mutated pUBI-βPS-Integrin-YFP transgenes in a background that was heterozygous for Integrin null mutant mysXG43, to ensure the overall Integrin level were similar to wild-type (Pines et al., 2011; Yuan et al., 2010). The Integrin mutants used
were as follows: L211I (Pines et al., 2011), G792N (Pines et al., 2011), N840A (Pines et al., 2011), N828A (Pines et al., 2012) and S196F (Pines et al., 2011). To combine ECM mutants with Integrin-YFP we introduced a copy of the pUBI-βPS-Integrin-YFP transgene into animals heterozygous for the mutants lan\textsuperscript{def} (Urbano et al., 2009), vkg\textsuperscript{k00236} (Spradling et al., 1999). The version of Rap1-DN used was Rap1-S17A (Ellis et al., 2013) driven by the muscle driver mef2GAL4 and combined with one copy of the pUBI-βPS-Integrin-YFP transgene.

To observe ECM proteins \textit{in vivo} we used endogenously GFP tagged versions of Laminin and Collagen IV (a gift from Frank Schnorrer, Max Planck Institute of Biochemistry, Germany) and an endogenously mCherry tagged version of Talin (Venken et al., 2011) to properly demarcate MTJs.

\textbf{2.2 FRAP experiments}

FRAP analysis was conducted in embryos (stage 16 and 17) and larvae (third instar). Embryos were dechorionated with 50% bleach for 4 min, and then embryos and larvae were washed with water. Animals are mounted in Phosphate-Buffered Saline (PBS). FRAP is performed after a waiting period (1-1.5h) in a diverse population of MTJs as described in Pines et al., 2013 with an inverted confocal microscope (Olympus Fluoview, FV1000) with an UplanSApo60x/1.35 oil objective (Olympus, Tokyo, Japan). Fluorescence intensity is measured in 825 frames, every 0.4 seconds and 75 frames were taken prior to the bleaching event. Bleaching is done using the Tornado scanning tool (Olympus) with a 473nm laser at 5% power during 2s at 100 μs/pixel. To control for drift of embryos, multiple regions of interest (ROIs) were selected in non-photobleached regions; only samples for which
intensities within control ROIs remained steady throughout the FRAP experiment were used. The mobile fraction was computed and statistical tests were performed using Prism 5 software.

2.3 Integrin activation assay

We used the protocol described in Schulman et al., 2013 in live stage 16 embryos to deliver MnCl$_2$ (20mM) and MgCl$_2$ (60mM) to ectopically activate Integrins. To determine if osmolarity could affect turnover, we used NaCl treated control embryos (60mM). After the treatment, embryos were mounted as previously described in halocarbon oil rather than PBS to prevent desiccation. FRAP was then performed as previously described, without the waiting period.

2.4 Endocytosis inhibition assay

We introduced the Dynamin dependent endocytosis inhibitor dynasore (200μM) using the protocol described in Schulman et al., 2013 in stage 17 embryos in the same conditions as the Integrin activation assay.

2.5 Mathematical modelling

To model Talin turnover, a three-compartment model was developed as a system of ordinary differential equations (see the Appendix) (Hakonardottir et al., 2015). To estimate model parameters, we fitted FRAP recovery curves to this model by minimizing the sum-of-square residuals between the model and the data. Confidence intervals on the parameters were estimated using bootstrapping (Efron and Tibshirani, 1993). To model Integrin
turnover, the data was fit to a two-compartment mathematical model that describes the dynamics of labeled Integrins cycling between the membrane and vesicles (see the Appendix). This mathematical model has been used in our previous work (Hakonardottir et al., 2015; Pines et al., 2012), and includes a correction for the background photobleaching of labels as in (Hakonardottir et al., 2015). Mobile fractions were used as an estimate of the fluorescence recovery fraction of each replicate. These were determined independently of the mathematical model and without photobleaching correction.
CHAPTER THREE: Results

3.1 In vivo quantitative analysis of Talin turnover in response to force

3.1.1 Development of a mathematical model and a modified FRAP approach to study Talin turnover

We previously described an analysis of Integrin turnover in cells exposed to different levels of mechanical force (Pines et al., 2012). This work relied on FRAP experiments, which, in combination with data fitting to a mathematical model, allowed the derivation of the two kinetic parameters that described Integrin turnover: the rate constant for the exocytosis, or delivery, of Integrins to the membrane \((k_{exo})\) and the rate constant for the endocytosis, or removal, of Integrins from the membrane \((k_{endo})\) (Figure 1.4e). To use a similar approach to study the turnover of the intracellular adhesion complex, we established a new mathematical model. The IAC component talin was chosen as the focus of these studies for a number of reasons: first, loss of Talin in flies fully recapitulates the phenotype obtained by loss of Integrins, implying that talin is essential for Integrin function, which is not the case for other components of the IAC in flies (Brown et al., 2002; Tanentzapf and Brown, 2006). Second, talin has been extensively studied in flies, and a large collection of tagged wild-type and mutant versions of talin is available for use (Ellis et al., 2013; Ellis et al., 2014; Ellis et al., 2011; Franco-Cea et al., 2010; Tanentzapf and Brown, 2006). Third, talin is a mechanosensory protein (del Rio et al., 2009), and its ability to sense and respond to force means that it is likely to play key roles in mechanotransduction through Integrin-
based adhesions. Fourth, talin mediates Integrin activation, a key mechanism for regulating Integrin adhesions in response to force (Calderwood et al., 1999). The FRAP analysis used a tagged genomic rescue construct, talin–GFP, which was shown to fully rescue the embryonic and larval talin loss-of-function phenotype and fully reproduce the expression of endogenous talin (Ellis et al., 2013; Ellis et al., 2014; Ellis et al., 2011; Franco-Cea et al., 2010; Tanentzapf and Brown, 2006). Talin-GFP is robustly expressed and has previously proven suitable for FRAP studies (Bouaouina et al., 2012; Ellis et al., 2014; Yuan et al., 2010).

A new mathematical model for turnover of IAC components was developed (Figure 3.1 a-c, Figure 3.11). The model assumes that the recruitment of Talin to the membrane occurs due to two simultaneous processes: the first is the delivery of Integrins and talin to the membrane, forming a new adhesion complex that includes talin as a core component; the rate constant that describes this process was therefore called \( k_{\text{assembly}} \) or \( k_{\text{asm}} \). Second, once a complex is assembled in the membrane, additional Talin can simply bind to the adhesion complex by interacting with any of its many known binding partners, by a rate constant we call \( k_{\text{on}} \). Next the model also assumes that the loss of talin from the membrane occurs due to two simultaneous processes: the first is the unbinding of talin from any of its binding partners from the adhesion complex, which itself remains intact; we call this rate constant \( k_{\text{off}} \). The second is the disassembly of the adhesion complex, as well as the internalization of Integrins and other components; this rate constant is \( k_{\text{disassembly}} \) or \( k_{\text{dis}} \). Talin turnover is therefore controlled by four parameters: \( k_{\text{on}}, k_{\text{off}}, k_{\text{dis}}, \) and \( k_{\text{asm}} \).

Use of this new mathematical model required adjustments to the protocol previously used to analyze Integrin turnover (Pines et al., 2012). First, because the new model has two
additional parameters that must be fitted, a greater amount of information must be collected in each experiment. This was accomplished in the FRAP protocol by increasing the rate of image acquisition. The new protocol gathered approximately eight times more data points per experiment than the previous protocol (see Materials and methods). When using the new protocol we observed a slight increase in overall levels of photobleaching due to the higher rate of scanning. FRAP curves shown have not been corrected for photobleaching; however, the data derived from the model (rate constants) were adjusted to include the effect of photobleaching (see Materials and methods). Second, when FRAP experiments were fitted to the model to obtain the four rate constants (Figure 3.1c, d), often there was more than one solution. This was because the model has a fundamental symmetry: without additional information, it is impossible to distinguish the association/dissociation pathway from the assembly/disassembly pathway (Figure 3.1b). This meant there were two symmetric sets of possible rate constants that allow the model to fit the graph. To overcome this challenge, we developed a “double-fitting” protocol (Figure 3.1d-f, Figure 3.8). For each experimental construct, we fitted the FRAP data to the model, thus obtaining two (symmetric) sets of rate constants describing the talin turnover dynamics. Next we repeated the FRAP experiments in the presence of an inhibitor of endocytosis (Figure 3.11) and fitted these data simultaneously with the unperturbed experiment under the assumption that only the rate constant for IAC disassembly ($k_{\text{dis}}$) would vary between the two conditions. This second experiment breaks the symmetry inherent in the model and allows us to obtain a single-valued estimate for each rate constant (Figure 3.1g). This technique proved highly efficient and informative (Figure 3.1d-f) and was carried out for each of the wild-type and mutant talin transgenes. The inhibition protocol relies on previous studies showing that the disassembly of Integrin-based adhesions requires endocytosis of Integrins (Ezratty et al.,
This meant that suppressing $k_{\text{dis}}$ could be accomplished by blocking endocytosis. To block endocytosis, a protocol was used to introduce the chemical inhibitor dynasore into live fly embryos (Schulman et al., 2013). The combination of the new model, improved time-resolution FRAP protocol, and double-fitting technique allowed us determine $k_{\text{on}}$, $k_{\text{off}}$, $k_{\text{dis}}$, and $k_{\text{asm}}$ for each set of FRAP experiments.

### 3.1.2 Analyzing the turnover of Talin under increased force

As previously shown, the amount of force that acts on Integrin-based adhesion in the MTJ can be increased in an inducible manner using the temperature-sensitive mutation $Brkd^{J29}$ (Montana and Littleton, 2004; Pines et al., 2012). Under wild-type conditions, beginning at early stage 16, muscles begin to undergo contractions that apply mechanical force on the MTJs (Crisp et al., 2008). However, induction of $Brkd^{J29}$ significantly increases both the amplitude frequency of muscle contractions and magnitude of the force generation compared with wild-type (Pines et al., 2012). Using an established temperature-shift protocol (Pines et al., 2012) (see Materials and methods), we exposed MTJs in stage 17 embryos containing the $Brkd^{J29}$ mutation to increased force and analyzed talin turnover using FRAP. In line with previous observations, the shift from 25°C to the non-permissive temperature of 37°C did not affect the mobile fraction of WT-talin-GFP in control embryos (Figure 3.2a, a’). In comparison, the application of force on the MTJs after a temperature shift led to a substantial, nearly 50% reduction in the Talin mobile fraction (Figure 3.2b, b’). This indicates that talin is stabilized at the membrane in response to the application of additional force on the adhesion complex.
Next we applied the “double-fitting” protocol to WT-talin-GFP and determined the rate constants $k_{\text{on}}$, $k_{\text{off}}$, $k_{\text{dis}}$, and $k_{\text{asm}}$. Similar to previous observations of Integrin, the temperature shift by itself affected the rate constants, probably due to a change in metabolic rates (Figure 3.2a’’). Moreover, the value of $k_{\text{asm}}$ at 25°C was somewhat higher in the Brkd$^{J29}$ mutant, likely due to the different genetic background and perhaps the possibility that even at the permissive temperature, the Brkd$^{J29}$ phenotype was manifested slightly. Of importance, the shift to the higher temperature in the Brkd$^{J29}$ mutant background affected some rate constants very differently compared with the wild-type control, indicating a robust and specific mutant phenotype (Figure 3.2b’’, c). Specifically, whereas in both the wild-type controls and Brkd$^{J29}$ mutants the temperature shift resulted in an increase in $k_{\text{on}}$ and $k_{\text{off}}$ and a slight decrease in $k_{\text{dis}}$, $k_{\text{asm}}$ was affected differently in controls versus mutant. In wild-type controls, $k_{\text{asm}}$ decreased by 14% upon temperature shift, but it increased by 263% in the Brkd$^{J29}$ mutants (Figure 3.2b’, c, c’’). To explore the effects of this shift on turnover, we calculated the ratio of the rate of adhesion complex assembly to disassembly (Figure 3.2d). Whereas in controls this ratio remained fairly constant at higher temperature (3.88 vs. 3.84 in 25 vs. 37°C, respectively) it increased nearly sevenfold in the induced Brkd$^{J29}$ mutants (1.28 vs. 8.32 in 25 vs. 37°C, respectively), consistent with a large shift toward a higher rate of adhesion complex assembly (Figure 3.2d). In comparison, the ratios of the rate of Talin binding on and off the assembled adhesion complex ($k_{\text{on}}/k_{\text{off}}$) were not substantially different at 25 vs. 37°C between the control and Brkd$^{J29}$ mutant (Figure 3.2c, d). Overall these data suggest that the main way in which talin becomes stabilized in response to force is through an increased rate of assembly of the adhesion complex.
3.1.3 Resolving the mechanism by which the turnover of Talin is regulated by increased force

To gain mechanistic insight into what regulates the rate of assembly of the Integrin adhesion complex in response to force, we used a well-characterized collection of targeted mutations in Talin that have been generated and analyzed in our lab (Table 3.1) (Ellis et al., 2014; Ellis et al., 2011; Franco-Cea et al., 2010). The effects of the BrkdJ29 mutation on the overall mobile fraction and the rate constants were analyzed in a background of Talin mutations that impinge on key aspects of talin function (Figure 3.3). Talin mediates three main functions: regulating Integrin activity, helping assemble and maintain the Integrin adhesion complex, and binding to actin. Mutations were chosen that interfered with each of these processes. One of the main ways talin is believed to modulate the stability of Integrin-based adhesion is by regulating Integrins through “inside-out” activation (Calderwood et al., 1999). A mutation (talinL334R; (Ellis et al., 2014)) that specifically blocks “inside-out” activation was previously introduced into a GFP-tagged genomic rescue construct for talin. GFP-tagged rescue transgenes were also generated that contain point mutations that block the two Integrin-binding sites of Talin, IBS1 (talinR367A; (Tanentzapf and Brown, 2006; Tanentzapf et al., 2006)) and IBS2 (talinK2094D/S2098D; (Ellis et al., 2011)). The binding of talin to Integrin through IBS1 is implicated in inside-out activation, whereas binding through IBS2 is required to maintain the linkage of the IAC to Integrin (Ellis et al., 2011; Tanentzapf and Brown, 2006). Finally, a GFP-tagged transgene was also made containing a mutation in talin that blocks the ability of its C-terminal THATCH domain to bind actin and link the cytoskeleton to Integrin (K2450D/V2451D/K2452D; (Franco-Cea et al., 2010).
Of importance, in a heterozygous state such as that used in our experiments, none of these mutations affected muscle integrity.

Before analyzing the turnover of talin transgenes containing point mutations, we confirmed that the temperature shift required to induce the Brkd<sup>129</sup> mutation, from 25 to 37°C, did not affect the mobile fraction of the mutant talin-GFP in control embryos (Figure 3.9). Intriguingly, on their own, none of the talin mutations studied completely blocked the typical reduction in turnover, manifested as a lower mobile fraction, caused by increasing the force acting on the MTJ (Figure 3.3). Nonetheless, mutations that disrupted talin’s ability to activate Integrin, bind Integrin through its IBS2 domain, or bind actin via its C-terminal THATCH domain exhibited an attenuated response to the application of force on the MTJ, as judged by overall change in mobile fraction (Figure 3.3b, d, e, respectively). This shows that the mutations tested impinged on, but did not completely block, the ability of turnover to be modulated by increased force. In addition, we confirmed that simply increasing the temperature did not result in a statistically significant change in turnover for wild-type or mutant versions of talin. To gain further insight into the effects of the talin mutations, we used the model fits to analyze changes in the reaction rates in response to increased force. Strikingly, we found that none of the talin mutations tested exhibited the increase in $k_{asm}$ that was observed for the WT-talin-GFP upon induction of the Brkd<sup>129</sup> mutation phenotype (Figure 3.3f). Nonetheless, these mutations exhibited compensatory changes, such as a larger decrease in $k_{off}$ compared with control that explained their ability to partially modulate turnover in response to increased force (Figure 3.3b-f). Analysis of the ratios of $k_{off}/k_{on}$ and $k_{asm}/k_{dis}$ in the talin mutants provided further support for the hypothesis that they interfered with the response to increased force Figure
3.3g). For example, upon application of higher force, all talin mutants showed a smaller increase in \( k_{asm}/k_{dis} \) than controls (Figure 3.3g). This indicates that in the talin mutants, adhesion assembly is not favored as it is in the wild-type. Taken together, these results show that the stabilization of talin in response to increased force is achieved by increasing the rate of assembly of new adhesions. Moreover, this increase in the rate of assembly is dependent on the ability of talin to activate Integrin, reinforce the adhesion complex, and link to actin. In the absence of these mechanisms, talin can still be stabilized by other, compensatory mechanisms, such as by reducing its rate of unbinding from already assembled adhesion complexes. However, these compensatory mechanisms result in a less robust response to increased force.

**3.1.4 Analyzing the turnover of Talin under decreased force**

Next we characterized the turnover of the Integrin adhesion complex at MTJs that experience reduced force compared with wild-type. As we previously showed (Pines et al., 2012), the amount of force that acts on Integrin-based adhesion in the MTJ can be decreased in an inducible manner using the temperature-sensitive mutation \( para^{ts2} \). Induction of \( para^{ts2} \) phenotype is known to significantly decrease the amplitude and frequency of muscle contractions, as well as the overall magnitude of the force generated by the muscle (Pines et al., 2012). The established temperature-shift protocol differed from that used for \( Brkd^{j29} \), as the effect is much weaker in embryos but becomes pronounced in larval stages (Pines et al., 2012). Thus all experiments employing \( para^{ts2} \) were performed in third-instar larva. FRAP analysis was carried out using the WT-Talin-GFP in MTJs where the \( para^{ts2} \) mutation was induced by transferring larva from the permissive (25°C) to the
non-permissive (37°C) temperature (Figure 3.4). No change in the mobile fraction of WT-Talin GFP in was observed compared with control embryos upon induction of the para^{ts2} phenotype (Figure 3.4a, a’, b, b’). This was similar to what was previously observed for Integrin upon induction of para^{ts2} (Pines et al., 2012). However, despite the overall constant mobile fraction analysis, the parameter fits for the mathematical model showed a uniform, across-the-board increase in all four rate constants (Figure 3.4a-c). Specifically, whereas in control third-instar larva a shift to the 37°C causes an overall drop in the rate constants (Figure 3.4a’’), this did not take place in the para^{ts2} mutants (Figure 3.4b), representing a net increase in all rate constants compared with the control (Figure 3.4c). This suggests that even though the proportion of talin that undergoes turnover remains the same, this pool undergoes more rapid turnover. Consistent with this hypothesis, analysis of the ratios $k_{asm}/k_{dis}$ and $k_{on}/k_{off}$ showed only minor differences between the control and para^{ts2} larvae, which explains how increased rates of turnover can occur while the mobile fraction remains constant (Figure 3.4). Taken together, these results show that reducing the force that acts on MTJ leads to a coordinated increase in the rate constants that govern Talin turnover, while the overall mobile fraction of talin remains constant.

3.1.5 Uncovering the mechanism by which the turnover of Talin is regulated by decreased force

Next we analyzed the effect of para^{ts2} in a background of talin mutations that interfered with inside-out activation, actin binding, and adhesion complex assembly. First, as before, we confirmed that simply increasing the temperature did not result in a statistically significant change in turnover for wild-type or mutant versions of talin. Second,
the force acting on MTJs was reduced by moving the flies to the non-permissive temperature. On induction of the paralysis, the effects on mobile fraction were, as in the wild-type, negligible for all Talin mutations, with the exception of the mutation in the C-terminal Integrin-binding site (IBS2), for which a small reduction in mobile fraction was seen (Figure 3.5a-e). Analysis of the rate constants governing talin turnover in mutant backgrounds revealed that mutations in the actin-binding domain or those that affect activation acted similarly to wild-type talin (Figure 3.5f). One exception was the mutation in IBS2 that affects adhesion complex assembly and stability (Ellis et al., 2011) (Figure 3.5e, f). In the IBS2 background, inducing the para\textsuperscript{ts2} mutation led to a much larger increase in the rate constants, and these changes were not as evenly distributed as they are in the wild-type. As a result, the ratio $k_{asm}/k_{dis}$ nearly doubled upon induction of the para\textsuperscript{ts2} phenotype; this increase in the overall assembly of adhesion explains the stabilization of adhesion manifest by the lower mobile fraction observed in the IBS2 mutant. These results show that under conditions of reduced force, the coordinated increase in rate constants that allows turnover to increase but to maintain a stable pool of talin at the membrane is dependent on the activity of the IBS2 domain of talin.

3.1.6 Reducing FAK activity partially mimics the effect of increased force on Talin turnover

FAK is a key regulator of Integrin turnover in response to force (Pasapera et al., 2010; Seong et al., 2013; Wang et al., 2001; Wang and Ha, 2013). We therefore asked whether modulating FAK activity could reproduce some of the affects of increased force. To this end, we expressed a mutant version of FAK (Y430F; equivalent to the vertebrate
Y397F mutation) that severely impairs the autophosphorylation site and consequently the ability of FAK to be activated (Grabbe et al., 2004; Macagno et al., 2014; Tsai et al., 2008). We found that the expression of the FAK(Y430F) transgene resulted in a lower talin mobile fraction, consistent with stabilization of talin at MTJs (Figure 3.6). Analysis of the turnover rate constants revealed that modulating FAK activity had a statistically significant effect on some of the rate constants compared with the control (Figure 3.6a). Specifically, the expression of the FAK(Y430F) resulted in an increase in $k_{\text{on}}$ and $k_{\text{asm}}$ and a slight decrease in $k_{\text{dis}}$. Specifically, $k_{\text{dis}}$ decreased by 32% upon expression of the FAK(Y430F) transgene, whereas $k_{\text{on}}$ and $k_{\text{asm}}$ increased by 150 and 27%, respectively (Figure 3.6b). To explore the effects of these changes in the rate constants on turnover, we calculated the ratio of the rate of adhesion complex assembly to disassembly ($k_{\text{asm}}/k_{\text{dis}}$) and found it to increase by 89%, from 5.6 to 10.6 (Figure 3.6c). In comparison, the ratio of the rate of talin binding off and on for the assembled adhesion complex ($k_{\text{off}}/k_{\text{on}}$) decreased by 42%, from 0.061 to 0.035 (Figure 3.6b, c). Overall these data suggest that FAK is required to maintain the junction in a more dynamic state and that preventing FAK activation both promotes junction assembly and suppresses junction breakdown.

### 3.1.7 Changes in rate constants underlie developmental regulation of Talin turnover

Over the course of development, Integrin-based adhesions at the MTJs become increasingly stable as the mobile fraction of Integrin and IAC components, including talin, Tensin, and ILK, decreases (Figure 3.7a, b) (Yuan et al., 2010). Reduced turnover can assist in the reinforcement and the buildup of talin at the MTJs that occurs as development progress (Devenport et al., 2007; Yuan et al., 2010). To determine the mechanism
underlying this developmentally regulated stabilization of the IAC at MTJs, we carried out a series of FRAP experiments with talin-GFP, starting at embryonic stages 15–17 and then in first- and third-instar larvae. As before, FRAP data were analyzed by fitting to the mathematical model (Figure 3.7). This analysis identified two different mechanisms that mediated the stabilization of junctions in the embryo, as well a third, separate mechanism that operated in larva. The first embryonic mechanism was responsible for the large decline in turnover during the transition from stage 15 to stage 16 in embryogenesis, which is the time when muscle contractility begins. During this transition, all of the rate constants, $k_{\text{off}}$, $k_{\text{on}}$, $k_{\text{asm}}$, and $k_{\text{dis}}$, declined. The second embryonic mechanism was responsible for reducing turnover during the transition from stage 16 to stage 17, which is the time when muscle contractility gains in strength. During this transition, the rates of adhesion complex disassembly, $k_{\text{dis}}$, and of talin binding off of the adhesion complex, $k_{\text{off}}$, declined substantially (Figure 3.7d, e). The third, larval mechanism was responsible for the moderate decrease in turnover that occurs over larval life, which is a time when muscles undergo massive growth. During this transition, there was a notable increase in the rate of Talin binding to already assembled adhesion complexes, $k_{\text{on}}$, and a smaller increase in the rate of assembly of new adhesion, $k_{\text{asm}}$ (Figure 3.7c, f). Therefore we conclude that the stabilization of cell–ECM adhesions at the MTJs is accomplished by stage-specific modulation of the different processes that mediate junctional turnover. Although initially, when muscles begin to contract, adhesions are stabilized by an across-the-board reduction in rate constants, in later embryonic stages, the rates of both adhesion complex disassembly and unbinding of talin from the assembled complex decline. In contrast, in larval stages, the stabilization of cell–ECM adhesions at the MTJs is accomplished largely by increasing the binding rate of talin to the assembled adhesion complex.
### 3.1.8. Chapter 3.1 table

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<th>Mutation</th>
<th>Domain</th>
<th>Function</th>
<th>Reference</th>
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<tr>
<td>R367A</td>
<td>FERM-IBS1</td>
<td>Prevents Talin head binding to Integrin so as Integrin activation</td>
<td>(Ellis et al., 2011; Tanentzapf and Brown, 2006)</td>
</tr>
<tr>
<td>L334R</td>
<td>FERM-IBS1</td>
<td>Blocks Integrin activation but not binding to Integrin</td>
<td>(Ellis et al., 2014; Haling et al., 2011)</td>
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<tr>
<td>K2094D/S2098D</td>
<td>IBS2</td>
<td>Disrupts Talin rod binding to Integrin</td>
<td>(Ellis et al., 2011; Moes et al., 2007; Rodius et al., 2008)</td>
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<tr>
<td>K2450D/K2451D/K2452D</td>
<td>Actin-binding (THATCH)</td>
<td>Blocks actin binding</td>
<td>(Franco-Cea et al., 2010; Gingras et al., 2009)</td>
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**Table 3.1. Table of Talin point mutations that affect Talin function.**
3.1.9 Chapter 3.1 figures

Figure 3.1. **FRAP protocol and mathematical model to describe talin turnover.** (a) Example of embryonic MTJs in a talin-GFP–tagged embryo in vivo (a’–a”’) Representative images of a FRAP experiment, showing (a’) the initial conditions, (a’’) the bleaching event, and (a”’) recovery over time. (b) Mathematical model that describes talin kinetics using four parameters: $k_{\text{on}}$ and $k_{\text{off}}$ to measure the rate of free cytoplasmic talin binding and unbinding at existing adhesion site, respectively, and $k_{\text{asm}}$ and $k_{\text{dis}}$ to measure the rate of complexed talin assembly and disassembly at the membrane, respectively. $\delta$, photobleaching rate. (c) Recovery curve of raw data for WT-talin-GFP at 25°C, fit adjustment and photobleaching adjustment derived from the mathematical model. (d) Simple fit bootstrap distribution for $k_{\text{asm}}$ shows two possible solutions to the model. (e) Endocytosis inhibition using the chemical inhibitor dynasore decreases talin turnover. (f) Bootstrap distributions for a double fit between normal data sets and endocytosis inhibition data sets. The symmetry between $k_{\text{on}}$-$k_{\text{asm}}$ and $k_{\text{off}}$-$k_{\text{dis}}$ is broken by performing a double fit assuming $k_{\text{dis}}$ is reduced. (g) Final bootstrap distribution for $k_{\text{asm}}$. Scale bar, 5 μm.
Figure 3.2. Increased mechanical force at MTJs stabilizes cell–ECM adhesion by regulating talin turnover. FRAP analysis of stage 17 embryonic MTJs was performed to determine fluorescence recovery (a, b), final mobile fractions (a', b'), and rate constants $k_{on}$, $k_{off}$, $k_{dis}$, and $k_{asm}$ (a'', b'') for WT-talin-GFP (a) and WT-talin-GFP in a Brkd^{29} background (b) at 25°C (light blue and red) and 37°C (dark blue and red). Relative change in rate constants (c, c') and rate constant ratios (d) at 25 and 37°C for wild-type controls and Brkd^{29} mutant flies. Each data point in a and b represents the mean value of on average 20 separate FRAP experiments; FRAP curves shown have not been corrected for photobleaching. Error bars are SEM. Error bars in a', a'', b', and b'' are 95% confidence intervals. ns indicates p > 0.05; *p ≤ 0.05, **p ≤ 0.01, and ***p ≤ 0.001.
**BrkΔ29 (embryonic stage 17)**

**WT (Talin)**

- **a** Fluorescence recovery
- **a’** Mobile fraction

**Activation mutant (Talin*L334>R)**

- **b** Fluorescence recovery
- **b’** Mobile fraction

**IBS-1 mutant (Talin*R367>A)**

- **c** Fluorescence recovery
- **c’** Mobile fraction

**IBS-2 mutant (Talin*K2094>D/S2098>D)**

- **d** Fluorescence recovery
- **d’** Mobile fraction

**Actin binding mutant (Talin*K2450>D/V2451>D/K2452>D)**

- **e** Fluorescence recovery
- **e’** Mobile fraction

**f** Relative change in rate constants: \( \frac{k_{37°C}}{k_{25°C}} \)

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<td>-16%</td>
<td>-49%</td>
<td>NS</td>
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<td>53%</td>
<td>-58%</td>
<td>-28%</td>
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<tr>
<td>Actin binding mutant</td>
<td>NS</td>
<td>-48%</td>
<td>NS</td>
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**g** Rate constant ratios

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<th>( k_{sam}/k_{dis} )</th>
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<td>37°C</td>
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<tr>
<td></td>
<td>0.70</td>
<td>0.49</td>
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<tr>
<td>WT (temp. control)</td>
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<tr>
<td>Activation mutant</td>
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<td>0.66</td>
</tr>
<tr>
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<td>0.36</td>
<td>0.12</td>
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<tr>
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<tr>
<td>Actin-binding mutant</td>
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Figure 3.3. FRAP analysis of mutant versions of talin uncovers mechanisms that regulate adhesion turnover in response to force. FRAP analysis of stage 17 embryonic MTJs determines fluorescence recovery (a–e), final mobile fractions (a′–e′), and rate constants $k_{on}$, $k_{off}$, $k_{dis}$, and $k_{asm}$ (a″–e″) at 25°C (light colors) and 37°C (dark colors) for wild-type and mutant versions of GFP-tagged talin in a wild-type and Brkd$^{29}$ background. Relative change in rate constants (f) and rate constant ratios (g) at 25 and 37°C for wild-type and Brkd$^{29}$ backgrounds using wild-type and mutant versions of talin-GFP. Each data point in a–e represents the mean value of on average 20 separate FRAP experiments; FRAP curves shown have not been corrected for photobleaching. Error bars are SEM. Error bars in a′–e′ and a″–e″ are 95% confidence intervals. ns indicates $p > 0.05$; *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$. 
Figure 3.4. Decreased mechanical force at MTJs modifies Talin turnover. FRAP analysis of third-instar larval MTJs were performed to determine fluorescence recovery (a, b), final mobile fractions (a’, b’), and rate constants \(k_{on}, k_{off}, k_{dis}, \) and \(k_{asm}\) (a”, b”) for WT-talin-GFP (a) and WT-talin-GFP in a para\(^{ts2}\) background (b) at 25°C (light blue and red) and 37°C (dark blue and red). Relative change in rate constants (c, c’) and rate constant ratios (d) at 25 and 37°C for wild-type controls and para\(^{ts2}\) mutant flies. Each data point in a and b represents the mean value of on average 20 separate FRAP experiments; FRAP curves shown have not been corrected for photobleaching. Error bars are SEM. Error bars in a’, a”, b’, and b” are 95% confidence intervals. ns indicates \(p > 0.05\); ***\(p \leq 0.001\).
Figure legend on the next page.
Figure 3.5. The Talin IBS2 domain is essential to coordinate turnover in response to reduced mechanical force. FRAP analysis of third-instar larval MTJs determines fluorescence recovery (a–e), final mobile fractions (a′-e′), and rate constants $k_{on}$, $k_{off}$, $k_{dis}$, and $k_{asm}$ (a″-e″) for wild-type and mutant versions of GFP-tagged talin at 25°C (light colors) and 37°C (dark colors) in a wild-type and a $para^{ts2}$ background. Relative change in rate constants (f) and rate constant ratios (g) at 25 and 37°C for wild-type and mutant versions of talin-GFP in wild-type and $para^{ts2}$ backgrounds. Each data point in a–e represents the mean value of on average 20 separate FRAP experiments; FRAP curves shown have not been corrected for photobleaching. Error bars are SEM. Error bars in a′–e′ and a″–e″ are 95% confidence intervals. ns indicates $p > 0.05$; *$p \leq 0.05$ and ***$p \leq 0.001$. 
Figure 3.6. Reduced FAK activity affects Integrin turnover in a manner similar to increased force. FRAP analysis of stage 17 embryonic MTJs were performed to analyze turnover of talin-GFP in control (*mef2GAL4/+) flies vs. flies expressing the non-active FAK-Y430F mutant (a), final mobile fractions (a'), and rate \( k_{\text{on}} \), \( k_{\text{off}} \), \( k_{\text{dis}} \), and \( k_{\text{asm}} \) (a''). Relative change in rate constants (b, b') and rate constant ratios (c) from controls and FAK-Y430F–expressing flies. Each data point in a represents the mean value of on average 20 separate FRAP experiments; FRAP curves shown have not been corrected for photobleaching. Error bars are SEM. Error bars in a' and a'' are 95% confidence intervals. ns indicates \( p > 0.05 \); *\( p \leq 0.05 \) and ***\( p \leq 0.001 \).
Figure 3.7. Talin turnover is developmentally regulated through distinct mechanisms.

(a) FRAP analysis of WT-talin-GFP at progressive developmental stages: embryonic stages 15 (e15), 16 (e16), and 17 (e17) and first (L1) and third (L3) larval instars. (b) Mobile fraction for talin-GFP at progressive developmental stages. Rate constants $k_{on}$ (c), $k_{off}$ (d), $k_{dis}$ (e), and $k_{asm}$ (f) for talin-GFP at progressive developmental stages. These experiments were conducted using a copy of the talin-GFP transgene in a heterozygous null mutant of the talin gene ($rhea^{79}$). Each data point in a represents the mean value of on average 20 separate FRAP experiments; FRAP curves shown have not been corrected for photobleaching. Error bars are SEM. Error bars in b are 95% confidence intervals. ns indicates $p > 0.05$; *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$. 

WT Talin in development (heterozygous background)
Figure 3.8. The symmetry between rate constants is broken by performing a double fit. Example of bootstrap distributions for a double fit between normal datasets and endocytosis inhibition datasets for (a) $k_{on}$, (b) $k_{off}$, (c) $k_{asm}$ and (d) $k_{dis}$. The endocytosis fit overlaps the most in (a) with the green peak which selects the green solution as $k_{on}$. This breaks the symmetry between rate constants and chooses the green solution for all rate constants. The endocytosis fits also overlap with all the green solutions which ensure that the chosen solutions for the rates constants are right.
**Figure 3.9. Increased temperature does not alter Talin turnover in a wild-type background.** FRAP analysis of stage 17 embryonic MTJs were performed to determine fluorescence recovery (a-d) and final mobile fractions (a’-d’) at 25°C (light colours) and 37°C (dark colours) for mutant versions of GFP tagged talin in a wild-type background. Each data point in curves (a-d) represents the mean value of an average of 20 separate FRAP experiments; FRAP curves shown have not been corrected for photobleaching. Error bars are SEM. Error bars 2 in (a’-d’) are 95% confidence intervals. For all panels, ns indicates a p-value > 0.05, * indicates a p-value ≤ 0.05, ** indicates a p-value ≤ 0.01 and *** indicates a p-value ≤ 0.001.
Figure 3.10. Mathematical model that describes talin turnover. The extended mathematical model consists of a compartmental model describing the interactions between: free and fluorescently labeled cytosolic proteins Talin concentration \(P\), the Integrin adhesion complex concentration at the membrane \(Cm\), Talin Integrin binding complex concentration at the membrane \(Bm\) and inside the cell \(Bc\). Integrin concentration in the membrane \(Im\) and inside the cell \(Ic\) remains at the pre-bleaching equilibrium during the recovery phase, since the FRAP experiment does not disrupt the chemical equilibrium. Talin \(P\) will bind Integrins at the membrane \(Im\) at rate \(k^{e}_{on}\) and form bound complex \(Bm\). The Integrin-Talin complex \(Bm\) will then form a full IAC \(Cm\) at rate \(\beta\) or will dissociate from the IAC at rate \(\alpha\). We assume that Talin can unbind from Integrins at the membrane (from a fully formed IAC, or from an Integrin-Talin complex \(Bm\)) at rate \(k^{e}_{off}\). On the other hand, we assume the Talin-Integrin complex cannot be broken or formed inside the cell. The IAC will assemble and externalize at rate \(k^{asm1}\) when Talin binds Integrin right before externalization, or at rate \(k^{asm2}\) when the \(Bc\) complex gets externalized, along with the rest of the complex proteins. Similarly the IAC will disassemble due to internalization of Integrins with Talin disassociated at rate \(k^{dis1}\), or by maintaining Talin binding, at rate \(k^{dis2}\). The membrane Talin-Integrin complex \(Bm\) will internalize at rate \(k^{in2}\) without being disrupted, or at rate \(k^{in1}\) when Talin disassociation happens. \(Bm\) will be externalized either from \(Bc\) at rate \(k^{ex2}\) or by externalization of Integrin and intermediate binding of Talin at rate \(k^{ex1}\). Since FRAP doesn't affect the other IAC component concentrations, we will treat them as implicitly included in the parameters \(\beta, k^{asm1}\) and \(k^{asm2}\). For the same reason, we will assume any other chemical interaction is in steady state during the time of the experiment. The fluorescence has a background photobleaching rate that is not related to the dynamics of the system. We will correct the FRAP data by including a photobleaching parameter \(\delta\). Both the IAC and the membrane binding complex \(Bm\) will lose fluorescence by natural photobleaching at rate \(\delta\). This type of bleaching will only affect the proteins in the membrane.
Endocytosis inhibition (stage 16) - Dynasoe and CPZ

Endocytosis inhibition (stage 17) - Dynasoe

Figure legend on the next page.
Figure 3.11. Endocytosis inhibition reduces Talin turnover. (a) Fluorescence recovery of Talin-GFP for e16 embryos upon no treatment (black), DMSO 150µM treatment (light blue), DMSO with dynasore 150µM (dark blue), DMSO 200µM treatment (light pink), DMSO with dynasore 200µM (dark pink). (b) Fluorescence recovery of Talin-GFP for e16 embryos upon treatment with buffer (light grey), buffer and CPZ 10µM (medium gray), buffer and CPZ 100µM (dark gray) and buffer and CPF 280µM (black). Fluorescence recovery of (c) Talin-GFP, (d) Talin*L334R-GFP, (e) Talin*R367A-GFP, (f) Talin*KS>DD-GFP and (g) Talin* KVK>DDD-GFP upon treatment with DMSO (grey) and treatment with DMSO and dynasore (black) in e17 embryos. Each data point in curves (a-g) represents the mean value of on average of 20 separate FRAP experiments; FRAP curves shown have not been corrected for photobleaching.
3.2 In vivo regulation of Integrin turnover by outside-in activation

3.2.1 Outside-in activation regulates Integrin turnover by divalent cations stabilizes cell-ECM adhesion by decreasing Integrin turnover.

We hypothesized that outside-in Integrin activation regulates Integrin turnover. We induced Integrin activation in vivo by introducing the well-known Integrin activators Mn$^{2+}$ and Mg$^{2+}$ into embryos (stage 16). Afterwards, we fitted our FRAP analysis to the previously published mathematical model that describes Integrin turnover using the rate constants $k_{\text{endo}}$ and $k_{\text{exo}}$ (Pines et al., 2012). By calculating the $k_{\text{endo}}/k_{\text{exo}}$ ratio we can easily compare whether adhesions build up or break down.

First we introduced NaCl (60mM) to control for osmolarity and determined that Integrin turnover is not affected by the osmolarity changes (Figure 3.12a, a’). Furthermore, the rate constants derived from the mathematical model show that $k_{\text{endo}}$ remains unchanged and $k_{\text{exo}}$ increases slightly upon high osmolarity conditions, although the overall $k_{\text{endo}}/k_{\text{exo}}$ ratio stays relatively constant with respect to the buffer conditions (Figure 3.12a’’, e). In contrast, ectopic Integrin activation with MnCl$_2$ (20mM) decreases Integrin turnover (Figure 3.12b, b’). This indicates that Integrin activation stabilizes cell-ECM adhesion by reducing Integrin turnover. After analysing the rate constants, we deduced that the stabilization comes from a 16% reduction in the endocytosis rate of Integrin and a 26% increase in the exocytosis rate, which allows more Integrins to be present at the membrane and reinforces the adhesion (Figure 3.12b’’, d). Activation with Mg$^{2+}$ recapitulates the effect in turnover produced by Mn$^{2+}$ (Figure 3.12c, c’). In this case, cell-ECM adhesion stabilization is just due to a 28% reduction in endocytosis rate while $k_{\text{exo}}$ is not affected.
Thus, endocytosis reduction seems to be the common mechanism to regulate adhesion turnover upon Integrin activation. To gain insight into this shift on turnover, we analyzed the $k_{\text{endo}}/k_{\text{exo}}$ ratio (Figure 3.12e). Whereas in buffer alone and buffer with Na$^+$ this ratio remained constant (~0.32), it significantly decreases (by a 34%) upon Integrin activation with Mn$^{2+}$ or Mg$^{2+}$ (0.32 vs. ~0.21 in buffer and Na$^+$ vs. Mn$^{2+}$ and Mn$^{2+}$ respectively). Taken together, this data suggests that Integrin activation plays a key role regulating cell-ECM adhesion turnover through a reduction in Integrin endocytosis rate in vivo.

### 3.2.2 Activating Integrin mutants regulate turnover similarly to treatment with divalent cations.

We hypothesize that if chemical Integrin activation regulates turnover, genetic activation should recapitulate this effect. We performed FRAP analysis of YFP-tagged Integrins with point mutations previously characterized in the lab that induce Integrin activation (L211I and G792N) (Table 3.2). The L221I mutation induces Integrin activation by promoting the extension of the extracellular domain of Integrin, while G792N promotes activation by increasing Integrin clustering at the membrane (Pines et al., 2011). We found that Integrin turnover for both activating mutants decreases in comparison to WT (Figure 3.13a, a’, b, b’). Moreover, the rate constants showed the same trend obtained in Mn$^{2+}$ activation: $k_{\text{endo}}$ decreases and $k_{\text{exo}}$ increases (Figure 3.13a’’, b’’, c). This suggests that cell-ECM adhesion becomes stabilized upon Integrin activation not only by endocytosis reduction but also by an increase in exocytosis. This is clearly stated by the ratio $k_{\text{endo}}/k_{\text{exo}}$, which is reduced by 70% in the L211I mutant compared to the wild-type (from 0.66 in WT to 0.19 in L211I) and by 30% in the G792N mutant (from 0.66 in WT to 0.38 in G792N)
We believe that the L211I has a stronger effect because this mutation causes a direct activation phenotype by hyperextending the extracellular domain.

3.2.3 Integrin mutations that affect outside-in activation fail to regulate Integrin turnover upon treatment with divalent cations.

If outside-in activation regulates Integrin turnover, we predict that Integrin point mutations that induce or block activation would be insensitive to treatment with divalent cations. To test this, we employed YFP-tagged Integrins with point mutations previously characterized in the lab (Table 3.2). First, we analyzed the activating mutants L211I and G792N. Mobile fractions for both mutants remain unchanged upon exposure to Mn$^{2+}$ (Figure 3.14a, a’, b, b’). Despite of a slight increase in $k_{exo}$ for L211I (Figure 3.14a’’) and a decrease in both $k_{endo}$ and $k_{exo}$ for G792N (Figure 3.14b’’), $k_{endo}/k_{exo}$ ratios barely change with respect to WT (Figure 3.14g). Secondly, we performed the same analysis using mutations that block Integrin activation (N828A, N840A and S196F). N828A and N840A are mutations in the NPxY domain that block Integrin activation by disrupting the binding of Talin and Kindlin, respectively (Pines et al., 2012; Pines et al., 2011). Finally, S196F is a mutation that allows ECM ligand binding but blocks the resulting extension of the extracellular domain, thus impeding activation (Pines et al., 2011). All three activation-blocking mutants also fail to reduce the mobile fraction after divalent cation treatment (Figure 3.14c, c’, d, d’, e, e’). In case of N828A and N840A, the rate constants do not exhibit any change (Figure 3.14c’’, d’’) while in case of S196F, only $k_{exo}$ slightly increases (Figure 3.14e’’). Overall, the $k_{endo}/k_{exo}$ ratio in these mutants remains similar to WT (Figure 3.14g). This data supports the reliability of the activation assay and the Integrin point
mutations. Furthermore, Integrins mutants with already active Integrins or Integrins that cannot undergo activation fail to regulate turnover upon Mn$^{2+}$ treatment.

### 3.2.4 ECM reduction destabilizes cell-ECM adhesion by increasing Integrin turnover.

Given that Integrin activation regulates turnover, we wondered if inhibiting activation would produce the opposite effect on turnover. ECM ligands can induce Integrin activation by binding to its extracellular domain. Therefore, we performed FRAP analysis in mutant embryos with reduced ECM content (stage 17); specifically, we removed Collagen IV and Laminin. We measured turnover of Integrin-YPF expressed in a $vkg^{k00236}$ and $lan^{def}$ mutant background. In ECM mutants, Integrin turnover increases with respect to WT (Figure 3.15a, a’, b, b’). In both cases, $k_{exo}$ remains unchanged but $k_{endo}$ dramatically increases (48% for the Laminin mutant and 61% for the Collagen mutant) (Figure 3.15a”, b”, c). These results suggest that in the absence of sufficient ECM ligands, Integrin turnover is increased and thus cell-ECM adhesions are destabilized. Furthermore, ECM removal causes the opposite effect on Integrin endocytosis than Integrin activation. Therefore these results suggest that ECM ligands induce outside-in activation in fly MTJs.

### 3.2.5 Integrin turnover is developmentally regulated by Integrin activation.

Previous data from the lab demonstrated that Integrin turnover is developmentally regulated; however the mechanisms by which this occurs have yet to be discovered. Given that ectopic activation of Integrins recapitulates the response of Integrin turnover kinetics in response to mechanical force, we hypothesized that increased muscle activity during development could remodel cell-ECM adhesions by regulating Integrin activation. To
determine whether changes in Integrin activation could account for stage-dependent differences in Integrin turnover, we performed Integrin turnover developmental profiling at stages e16, e17 and L3 and extracted the rate constants for the mathematical model. We determine that stabilization of Integrins during development occurs via two mechanisms: the reduction of $k_{\text{endo}}$ between e16 and e17 stages (clearly stated by a 57% reduction in the $k_{\text{endo}}/k_{\text{exo}}$ ratio), and the increase in $k_{\text{exo}}$ between every developmental stage (Figure 3.16 and Figure 3.17 a-a’’, e).

To further test our hypothesis, we performed the developmental profiling using the activated Integrin mutants L211I and G792N. L211I embryos exhibit no change in turnover between stages e16 and e17 (Figure 3.16b-b’’). This is due to the failure to reduce $k_{\text{exo}}$ to the same extent as WT and a lack of increase in $k_{\text{endo}}$ (Figure 3.16b’’). Consequently, the $k_{\text{endo}}/k_{\text{exo}}$ ratio also is unchanged between stages (Figure 3.16e). G792N shows a similar but weaker effect to WT since the mobile fraction, rate constants, and constant ratio give a less strong effect than WT (Figure 3.16c-c’’).

The activation-blocking mutants N828A and S196F also fail to achieve the characteristic drop in turnover between embryonic stages (Figure 3.17c-c’, d-d’). This is caused by abnormalities in the endocytosis and exocytosis rates. The blocking mutant N840A gives the effect than WT but in a lesser extent. For all mutants, $k_{\text{endo}}/k_{\text{exo}}$ rate remains unchanged between stages e16 and e17 (Figure 3.17f). Based on this data, we propose that modulation of Integrin activation is important for the regulation of Integrin turnover during animal development.
3.2.6 *Rap1 regulates Integrin turnover downstream of outside-in activation.*

To gain mechanistic insight on how outside-in signalling is conducted, we next tested if outside-in signalling is transmitted downstream by the GTPase Rap1. Therefore we performed FRAP analysis after Mn^{2+} treatment in embryo expressing a Rap1-DN transgene as well as Integrin-YFP. Rap1-DN expression was driven in the muscles using the driver *mef2GAL4*. First, we observe that Rap1-DN does not interfere with Integrin turnover (Figure 3.18a, a’). Interestingly, Rap1-DN embryos fail to reduce turnover upon chemical activation (Figure 3.18a, a’). Both mobile fraction and rate constants remain unchanged after Mg^{2+} treatment (Figure 3.18a’, a’’, b). In summary, this data suggests that Rap1 regulates Integrin turnover downstream of outside-in activation.
### 3.2.7 Chapter 3.1 table

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<th>Domain</th>
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<td>N828A</td>
<td>Membrane-proximal NPxY</td>
<td><em>Blocks activation:</em> perturbs Talin binding</td>
<td>(Calderwood et al., 2002; Ellis et al., 2011; Pines et al., 2012; Tadokoro et al., 2003; Tanentzapf, 2006; Tanentzapf and Brown, 2006; Wegener et al., 2007)</td>
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<td>N840A</td>
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<td>S196F</td>
<td>ECM binding domain</td>
<td><em>Blocks activation:</em> binds ECM ligands but it is unable to undergo extracellular conformational changes</td>
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<td>L211I</td>
<td>I-like</td>
<td><em>Induces activation:</em> promotes extracellular domain extension</td>
<td>(Jannuzi et al., 2004; Luo et al., 2009; Pines et al., 2011)</td>
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<td>G792N</td>
<td>TM</td>
<td><em>Induces activation:</em> increases clustering</td>
<td>(Li et al., 2003; Pines et al., 2011)</td>
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Table 3.2. Table of Integrin point mutations that affect Integrin activation.
3.2.8 Chapter 3.2 figures

Osmolarity control (Na⁺) - WT

**a**  
β-integrin fluorescence recovery  
$n = 17\hspace{1cm}n = 30$

**a’**  
Mobile fraction

**a’’**  
Rate constant (s⁻¹)

**a’’’**  
Mobile fraction

Mn²⁺ treated embryos - WT

**b**  
β-integrin fluorescence recovery  
$n = 17\hspace{1cm}n = 15$

**b’**  
Mobile fraction

**b’’**  
Rate constant (s⁻¹)

**b’’’**  
Mobile fraction

Mg²⁺ treated embryos - WT

**c**  
β-integrin fluorescence recovery  
$n = 17\hspace{1cm}n = 16$

**c’**  
Mobile fraction

**c’’**  
Rate constant (s⁻¹)

**c’’’**  
Mobile fraction

**e**  
$k_{endo}/k_{exo}$ ratio

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<th>$k_{exo}$</th>
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</tr>
<tr>
<td>Mg²⁺ treatment</td>
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<td>NS</td>
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**Treatments** kendo/kexo

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<tr>
<td>Mn²⁺ treatment</td>
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<td>Mg²⁺ treatment</td>
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Figure legend on the next page.
Figure 3.12. Outside-in Integrin activation using divalent cations stabilizes cell-ECM adhesion by decreasing Integrin turnover. Fluorescence recovery (a-c), mobile fractions (a’-c’) and rate constants $k_{\text{endo}}$ and $k_{\text{exo}}$ (a’’-c’’) for WT Integrin-YFP treated with buffer (light green), buffer containing NaCl (a-a’’, dark green), buffers containing MnCl$_2$ (b-b’’, dark green) or MgCl$_2$ (c-c’’, dark green). (d) Relative change in rate constants between buffer only control and buffer containing salts. (e) $k_{\text{endo}}/k_{\text{exo}}$ ratio for each experimental group. All FRAP experiments were done on embryonic MTJs (stage 16), using a copy of the Integrin-YFP transgene in a heterozygous Integrin null mutant (mys$^{XG43}$) background. All data points in curves are the mean of an average of 15 separate FRAP experiments and error bars are SEM. Error bars in (a’-c’) are 95% confidence intervals. Error bars in (a’’-c’’) are 95% bootstrap confidence intervals. For all panels: ns means a p-value > 0.05, * means a p-value ≤ 0.05, ** means a p-value ≤ 0.01, *** means a p-value ≤ 0.001.
WT vs Activating integrin mutants

Figure 3.13. Point mutations in Integrin that induce activation affect turnover similarly to treatment with divalent cations. Fluorescence recovery (a, b), mobile fractions (a’, b’) and rate constants $k_{\text{endo}}$ and $k_{\text{exo}}$ (a’’, b’’) for WT Integrin-YFP (black) and YFP tagged mutant versions of Integrin (maroon). (c) Relative change in rate constants between WT and activated Integrin mutants. (d) $k_{\text{endo}}/k_{\text{exo}}$ ratio for each experimental group. All FRAP experiments were done on embryonic MTJs (stage 16) and using a copy of the Integrin-YFP transgene in a heterozygous Integrin null mutant (mysXG43) background. All data points in curves are the mean of an average of 15 separate FRAP experiments and error bars are SEM. Error bars in (a’-b’) are 95% confidence intervals. Error bars in (a’’-b’’) are 95% bootstrap confidence intervals. For all panels: * means a p-value ≤ 0.05, *** means a p-value ≤ 0.001.
**Figure legend on the next page.**
Figure 3.14. Mutations that block or strongly induce outside-in Integrin activation are insensitive to treatment with divalent cations. Fluorescence recovery (a-e), mobile fractions (a’-e’) and rate constants $k_{\text{endo}}$ and $k_{\text{exo}}$ (a’’-e’’) for YFP tagged mutant versions of Integrin treated with buffer only (light red and light blue) or with buffer containing MnCl$_2$ (dark red and dark blue) for activated Integrin mutants (red) or activation deficient Integrin mutants (blue). (f) Relative change in rate constants between buffer only control and buffer containing added salts. (g) $k_{\text{endo}}/k_{\text{exo}}$ ratio for each experimental group. All FRAP experiments were done on embryonic MTJs (stage 16) and using a copy of the Integrin-YFP transgene in a heterozygous Integrin null mutant ($\text{mys}^{XG43}$) background. All data points in curves are the mean of an average of 15 separate FRAP experiments and error bars are SEM. Error bars in (a’-e’) are 95% confidence intervals. Error bars in (a’’-e’’) are 95% bootstrap confidence intervals. For all panels: ns means a p-value $> 0.05$, * means a p-value $\leq 0.05$, *** means a p-value $\leq 0.001$. 
Figure 3.15. Reducing the availability of ECM ligands increases Integrin turnover. Fluorescence recovery (a-b), mobile fractions (a’-b’) and rate constants $k_{\text{endo}}$ and $k_{\text{exo}}$ (a’’-b’’) for WT Integrin-YFP in a WT background (light orange) and WT Integrin-YFP in the background of a heterozygous mutant for the genes encoding β Laminin subunit (a-a’, lan$^{\text{def}}$) or Collagen IV (b-b’, vkg$^{\text{K00236}}$) (dark orange). All FRAP experiments were done on embryonic MTJs (stage 17). (c) Relative change in rate constants between the WT and the heterozygous ECM mutants. (d) $k_{\text{endo}}/k_{\text{exo}}$ ratio for each experimental group. All data points in curves are the mean of an average of 15 separate FRAP experiments and error bars are SEM. Error bars in (a’-b’) are 95% confidence intervals. Error bars in (a’’-b’’) are 95% bootstrap confidence intervals. For all panels: ns means a p-value > 0.05, * means a p-value ≤ 0.05, ** means a p-value ≤ 0.01, *** means a p-value ≤ 0.001.
Integrin turnover at different developmental stages - WT

Integrin turnover at different developmental stages - Activating integrin mutants

d Relative change in rate constants

e \(k_{\text{endo}}/k_{\text{exo}}\) ratio

---

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Figure 3.16. Integrin turnover is developmentally regulated by Integrin activation – analysis of activating mutants. Fluorescence recovery (a-c), mobile fractions (a’-c’) and rate constants k_{endo} and k_{exo} (a”-c”’) for WT Integrin-YFP (a) and activated Integrin mutants (b, c) at progressive developmental stages: embryonic stages 16 (e16, green) and 17 (e17, purple) and third larval instar (L3, blue). (d) Relative change in rate constants between e16 and L3 for all genotypes. (e) k_{endo}/k_{exo} ratio for each experimental group. All FRAP experiments were done using a copy of the Integrin-YFP transgene in a heterozygous Integrin null mutant (mys\textsuperscript{XG43}) background. All data points in curves are the mean of an average of 15 separate FRAP experiments and error bars are SEM. Error bars in (a’) are 95% confidence intervals. Error bars in (a’’) are 95% bootstrap confidence intervals. For all panels: ns means a p-value > 0.05, * means a p-value ≤ 0.05, ** means a p-value ≤ 0.01, *** means a p-value ≤ 0.001.
Integrin turnover at different developmental stages - WT

![Graphs showing fluorescence recovery over time for different genotypes](image)

**Integrin turnover at different developmental stages - Activation blocking integrin mutants**

![Graphs showing fluorescence recovery over time for different genotypes](image)

**e** Relative change in rate constants

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**f** $k_{endo}/k_{exo}$ Ratio

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**Figure 3.17. Integrin turnover is developmentally regulated by Integrin activation – analysis of activation blocking mutants.** Fluorescence recovery (a-e), mobile fractions (a’-e’’) and rate constants $k_{endo}$ and $k_{exo}$ (a’’-e’’’) for wildtype (a) and YFP tagged mutant versions of Integrin (b-d) at progressive developmental stages: embryonic stages 16 (e16, green) and 17 (e17, maroon) and third larval instar (L3, blue). (e) Relative change in rate constants between e16 and L3 for all genotypes. (f) $k_{endo}/k_{exo}$ ratio for each experimental group. All FRAP experiments were done using a copy of the Integrin-YFP transgene in a heterozygous Integrin null mutant ($mys^{XG43}$) background. All data points in curves are the mean of an average of 15 separate FRAP experiments and error bars are SEM. Error bars in (a’-d’) are 95% confidence intervals. Error bars in (a’’-d’’) are 95% bootstrap confidence intervals. For all panels: ns means a p-value $> 0.05$, * means a p-value $\leq 0.05$, ** means a p-value $\leq 0.01$, *** means a p-value $\leq 0.001$. Wild-type data set shown is the same as that shown in Figure 3.2.5 and is included as a comparison to the mutant data.
Figure 3.18. Rap1 regulates Integrin turnover downstream of outside-in activation. Fluorescence recovery (a, b), mobile fractions (a’, b’) and rate constants k_endo and k_exo (a’’, b’’) for WT Integrin-YFP in a WT background (a-a’’, b-b’’, black) or in flies expressing a Rap1 dominant negative transgene (Rap1-DN) in embryonic muscles treated with buffer (a-a’’, light pink) or buffer with MnCl_2 (a-a’’, dark pink) (c) Relative change in rate constants between WT, mutant and mutant treated with mutants MnCl_2. (d) k_endo/k_exo ratio for each experimental group. All FRAP experiments were done on embryonic MTJs (stage 16) and using a copy of the Integrin-YFP transgene in a heterozygous Rap1-DN background expressed in the muscle using a mef2 promoter. All data points in curves are the mean of an average of 15 separate FRAP experiments and error bars are SEM. Error bars in (a’-b’’) are 95% confidence intervals. Error bars in (a’’-b’’) are 95% bootstrap confidence intervals. For all panels: ns means a p-value > 0.05.
3.3 *In vivo* analysis of ECM components turnover

3.3.1 The major ECM proteins, Collagen and Laminin, undergo turnover

In this thesis and in our lab’s previous work, we investigated the various mechanisms by which cell-ECM adhesion protein turnover is regulated (Pines et al., 2012; Yuan et al., 2010). However, we have not yet addressed the behavior of a major component of cell-ECM adhesion, namely the ECM itself. Since Integrin and the IAC components undergo turnover, we hypothesized that ECM components undergo turnover *in vivo* too. FRAP analysis was carried using GFP tagged versions of Collagen IV and of the alpha and beta Laminin subunits (LanA and LanB1). Since ECM proteins have a wide expression pattern throughout the embryo, we identified ECM at MTJs by double-labelling with Talin-mCherry. Additionally, we determined that the mCherry signal does not interfere with the GFP signal during the FRAP analysis by performing FRAP with only Vkg-GFP (Figure 3.19a). Preliminary data demonstrates that Collagen IV and Laminin undergo turnover (Figure 3.19a, b, c).

3.3.2 Laminin ECM is remodelled upon Integrin activation

The next question we addressed is whether or not ECM turnover is regulated in a similar way as Integrin turnover, for example by Integrin activation. Interestingly, LanA turnover increases upon Integrin activation (Figure 3.19d). This suggests the Laminin ECM network is remodelled in response to activation of Integrins.
3.3.3 Chapter 3.3 figures

**ECM proteins turnover**

**Vkg fluorescence recovery**

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<th>Time (s)</th>
<th>Fluorescence intensity (normalized)</th>
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**Fluorescence control**

- $n = 11$
- $n = 14$

**LanA fluorescence recovery**

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**Fluorescence control**

- $n = 15$

**LanB1 fluorescence recovery**

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**Fluorescence control**

- $n = 10$

**Laminin turnover upon integrin activation**

**LanA fluorescence recovery**

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**Fluorescence recovery of (a, light green) Vkg-GFP** in absence of Talin-mCherry. This acts a control to determine that the red signal does not interfere with GFP signal during FRAP analysis.

**Figure 3.19. Collagen IV and Laminin undergo turnover in vivo.** Fluorescence recovery of (a, dark green) Vkg-GFF, (b) LanA-GFP and (c) LanB-GFP1. MTJs were demarcated by expressing a copy of a Talin-mCherry endogenous transgene. (a, light green) Fluorescence recovery of Vkg-GFP in absence of Talin-mCherry. This acts a control to determine that the red signal does not interfere with GFP signal during FRAP analysis. (d) Fluorescence recovery of LanA-GFP (MTJs demarcated by Talin-mCherry) treated only with buffer (light blue), buffer containing MnCl$_2$ (dark blue).
CHAPTER FOUR: Discussion

4.1 *In vivo* quantitative analysis of Talin turnover in response to force

In this thesis, we first assessed the effect of mechanical force on the turnover of the IAC protein Talin *in vivo* using a combination of genetics and quantitative analysis. Specifically, we employed the temperature sensitive mutants *Brkd*\textsuperscript{J29} and *para*\textsuperscript{ts2} to manipulate mechanical force by altering muscle contraction. We then used a mathematical model to quantify Talin’s turnover kinetics. Our results confirm that elevated force applied to adhesion sites stabilizes cell-ECM adhesion by decreasing adhesion complex turnover. This regulation comes from a substantial increase in the rate of Talin assembly as a complex. In contrast, force reduction on the MTJs does not alter the proportion of Talin at the MTJs, although Talin is recruited in a different manner than wild-type. We further demonstrate that as embryos develop and tissue architecture is consolidated, cell-ECM adhesion becomes more stable by decreasing Talin turnover. Taken together, this data provides *in vivo* mechanistic insight into how cell-ECM adhesion stability mediates animal development.

It has been previously shown that mechanical force is able to reinforce new adhesions (Lee et al., 2013; Puklin-Faucher and Sheetz, 2009). Elegant work on Talin conformation demonstrated that upon applied force, the Talin rod undergoes conformational changes that expose cryptic protein-protein binding sites (del Rio et al., 2009). From here, it has been hypothesized that the recruitment of IAC proteins, such as vinculin, to the newly exposed cryptic binging sites of Talin triggers a signalling event.
leading to the stabilization of the adhesion. Our research strongly supports this idea. Upon increased mechanical force, cell-ECM adhesion becomes quickly reinforced by a considerable increase in the rate of adhesion complex assembly. In line with this, adhesion complex assembly fails to stabilize cell-ECM adhesion upon increased force when utilizing mutant forms of Talin that abrogate important functions of the protein. Furthermore, previous work from our lab has showed that mechanical force increases Integrin availability at the membrane by reducing the endocytosis rate of Integrin. In contrast to this, mechanical force reduction did not alter the proportion of Talin undergoing turnover (the mobile fraction remains unchanged), nevertheless, it modifies the way Talin is recruited to the adhesion sites (Talin rate constants vary upon force reduction). This agrees with the work published in Pines et al., 2012 where Integrin turnover remains unchanged upon decreased force conditions despite of changing Integrin endocytosis and exocytosis rates. These findings suggest that there is a similar mechanism by which Integrin and Talin turnover respond to decreased mechanical force.

From our mutational analysis, we inferred that the only process that seems to be directly affecting mechanical force-originated signal transduction is Integrin activation. The Talin mutant L334R, which binds Integrin but lacks the abilities to activate it, fails to decrease turnover and to induce adhesion complex assembly upon increased mechanical force. This result implies that force-dependent regulation of cell-ECM adhesion turnover requires Integrin activation. Furthermore, it is important to mention that the mutational analysis also elucidates an important role for the IBS2. Previous work in flies demonstrated that the IBS2 is involved in strengthening of cell-ECM adhesions (Ellis et al., 2011).
Moreover, *in vitro* FRAP studies demonstrated that Talin partner’s kinetics is regulated by IBS2 (Himmel et al., 2009).

We also characterized the role of Talin turnover over the course of fly development. We found gradual cell-ECM stabilization throughout development, which is manifested by a reduction of Talin turnover. There are three major regulation points when turnover significantly drops: mid embryogenesis, late embryogenesis and larval stage. From e15, where muscles are not yet developed, to e16, where the body plan of the embryo is established, all the rate constants decrease, indicating stabilization of cell-ECM adhesion. In e17, as the muscles contract due to locomotion, turnover decreases by decreasing Talin removal to the membrane (k_{dis} and k_{off}). In contrast, larval stabilization of cell-ECM adhesion relies on increasing the delivery of Talin to the adhesion sites (k_{asm} and k_{on}), as seen when embryos were subjected to high mechanical force conditions. The fact that in the transition between e16 and e17 stages Talin turnover does not rely on the k_{asm} suggests that mechanical force is not the only factor regulating turnover. Moreover, several *in vitro* studies support the idea that mechanical force is not always sufficient for the maturation of FAs (Oakes et al., 2012; Oakes and Gardel, 2014; Stricker et al., 2011). Given our results with the activation deficient mutant L334R, we believe that activation may also serve as a developmental regulator of cell-ECM adhesion.

Taken together, we propose that cell-ECM adhesion stability depends on the regulation of Talin turnover by mechanical force. As force increases, we believe the concentration of Integrin and Talin at the membrane builds up thanks to a reduction in the removal of Integrin by endocytosis and a rapid increase in adhesion complex assembly. We further show that Integrin activation plays an important role in controlling cell-ECM
adhesion turnover as result of mechanical force application and developmental stabilization, since the activation mutant L334R fails to regulate turnover upon high force conditions.

4.2 In vivo regulation of Integrin turnover by outside-in activation

Previous studies in Drosophila have shown that Talin-mediated Integrin activation is important for development and homeostasis (Tanentzapf and Brown, 2006). However, certain publications suggest that Integrin activation vary between flies and vertebrates (Helsten et al., 2008). Recent work using the fly Talin mutant L334R, which abrogates inside-out activation but not Integrin binding, did not find an abnormal embryonic phenotype (Ellis et al., 2014). Moreover, Mn\(^{2+}\) and Mg\(^{2+}\) treatment but not Talin-mediated inside-out activation induced an increase in ECM ligand binging affinity in Drosophila S2/M3 cells (Helsten et al., 2008). Taken together, this evidence suggests that outside-in activation might have a stronger role than inside-out activation in regulating cell-ECM adhesion in Drosophila. Therefore, we explored the role of outside-in activation during fly embryogenesis.

Using chemical and genetic approaches in combination with mathematical modelling, we analyzed the effect of outside-in Integrin activation on Integrin turnover in vivo. We modulated Integrin activity in three way: (1) using divalent cations known to activate Integrins extracellularly, (2) using embryos expressing activated Integrin mutants and (3) by genetically reducing the amount of available ECM ligands. Our results suggest that cell-ECM adhesion is stabilized upon outside-in activation by reducing Integrin turnover. Specifically, this drop in mobile fraction is due to a decrease in the rate of Integrin endocytosis and an increase in Integrin exocytosis. We further demonstrate that as
muscles mature, cell-ECM adhesion becomes more stable; we propose that this transition is due to the control of turnover by outside-in activation. Taken together, this data links Integrin activation to Integrin turnover as vital modulators of cell-ECM adhesion stability in vivo.

Previous literature has described some links between Integrin activation and trafficking in vitro. However, active β1 Integrins can also be endocytosed, but by a different mechanism than the inactive Integrins (Valdembri and Serini, 2012). The Ivaska lab studied endocytic traffic of active β1 Integrin using Integrin antibodies that target specific conformation of the protein. They found that active β1 Integrins are endocytosed at a faster rate than inactive Integrins (Arjonen et al., 2012). On the other hand, during Integrin exocytosis to the membrane, Ca$^{2+}$ binding prevents outside-in Integrin activation (Tiwari et al., 2011). Additionally, exocytosis is related to turnover by the differential sorting of β1 Integrins as they are generally a target of degradation, but if sorting nexin 17 leaves the Integrin cytoplasmic tail, β1 Integrins are recycled to the plasma membrane (Bottcher et al., 2012; Bunch et al., 1992).

Interestingly, our results are very much in line with previous results describing Integrin turnover in vivo. Work form the Tanentzapf lab showed that mechanical force stabilizes Integrin turnover in part using similar mechanisms as Integrin activation, by reducing $k_{\text{endo}}$ (Pines et al., 2012). Moreover, from the Talin FRAP analysis we deduced that Integrin activation mediates turnover regulation by mechanical force. Together, these results propose a link between force and activation where cell-ECM adhesion stabilization via mechanical force is mediated by outside-in activation.
Moreover, as previously mentioned, mechanical force is not the only regulatory mechanism of cell-ECM stability throughout development. Here we demonstrate that Integrin activation plays a key role in the transition between tissue morphogenesis and maintenance. The major regulatory step in development occurs in the embryonic stage 17 and it is due to a reduction in Integrin $k_{\text{endo}}$ and an increase in $k_{\text{exo}}$.

### 4.3 *In vivo* analysis of ECM components turnover

Finally, we demonstrated that the major ECM components, Laminin and Collagen IV, undergo turnover in the context of a developing embryo. Additionally, our preliminary data suggests that Laminin A turnover increases after Mn$^{2+}$ treatment. This data suggests that the ECM is remodelled upon Integrin activation. Several *in vitro* studies support this finding. It is known that Integrin activation induces ECM formation in 2D cultures (Humphries et al., 2004). In line with this, activation of Integrins using urokinase-type plasminogen activator receptor (uPAR) ligands, known to control $\alpha_5\beta_1$ activation, leads to fibronectin ECM assembly (Monaghan et al., 2004). Overall, these findings in combination with ours imply that Integrin activation (induced by exogenous factors like Mn$^{2+}$ or uPAR ligand) promotes ECM turnover and in turns ECM production *in vitro* and *in vivo*.

### 4.4 Model of cell-ECM adhesion turnover regulation throughout development

Taken together, our research suggests that consolidation of tissue architecture is regulated by modulation of cell-ECM adhesion components turnover. We propose the following model: as the muscles grow during embryogenesis, they begin to contract, applying more mechanical force on MTJs (Crip et al., 2008). Increased mechanical force
produces reinforcement of the Integrin adhesion complex at the membrane (by reducing the Integrin endocytosis rate) via outside-in activation. Moreover, the enlarged concentration of Integrin at the membrane helps the assembly of Talin as a complex (Hakonardottir et al., 2015). Next, outside-in signalling contributes to cell-ECM adhesion reinforcement by increasing Integrin $k_{\text{exo}}$. Rap1 could potentially be activated as a consequence of the signalling event and induce Integrin trafficking, leading to an increase in Integrin exocytosis (Caswell and Norman, 2006; Medeiros et al., 2005). Finally, as a consequence of outside-in activation, ECM is remodelled by increasing ECM turnover and production in order to modulate cell-ECM adhesion stability. To conclude, this mechanism provides a framework for translating environmental cues such as the availability of ECM ligands or the mechanical forces present in a developing tissue into changes in the stability of Integrin-based cell-ECM adhesion.

### 4.5 Caveats

One of the caveats of this research is the use ubiquitously expressed Talin and Integrin transgenes as it has the potential to alter levels of expression. However, these transgenes have been shown to localize normally and rescue phenotypical defects in a null mutant background (Ellis et al., 2011; Franco-Cea et al., 2010; Pines et al., 2012; Pines et al., 2011; Tanentzapf and Brown, 2006). Furthermore, ideally all of these experiments would have been done in a heterozygous null mutant background for Talin or Integrin to eliminate competition between the fluorescently tagged protein and the endogenous ones. However, for experimental simplicity, some of the experiments were performed in a WT background. This might have given a less intense fluorescent signal. Finally, the
endocytosis inhibitor dynasore is a potent drug that could have given some off target effects (for example, blockage of other pathways necessary for the normal functioning of Integrin turnover and the cell).

4.6 Future directions

From these results, several questions arise and remain: What is the mechanism by which outside-in activation regulates Integrin traffic? What is the exact machinery that regulates differential turnover of active and inactive Integrins? What is the specific role of inside-out activation to cell-ECM adhesion modulation? To address them I propose further characterization of *Drosophila* inside-out and outside-in Integrin activation. We already established that outside-in activation is a crucial process for fly morphogenesis. To explore in depth the mechanism by which outside-in activation mediates embryogenesis, I propose the development of antibodies that specifically target active or non-active versions of Integrins. More controversial is the contribution of inside-out activation to cell-ECM adhesion modulation. We previously discussed that Talin mediated inside-out activation does not seem to be a major regulator (Section 4.2). However, we know from *in vitro* studies that the IAC protein Kindlin participates in inside-out activation as a co-activator. It is possible that Kindlin-based Integrin activation is an important regulator of Integrin-based adhesion in flies. I propose to elucidate how Kindlin contributes to this process by performing structure-function and turnover analysis in the *Drosophila* embryos using GFP-tagged wild-type and functional mutants of Kindlin. In addition, the Talin turnover mathematical model could be applied to Kindlin, since the ways in which they can be recruited to Integrins are similar. Another exciting future direction for this project is the
investigation of the role of ECM ligand turnover in fly morphogenesis. Our preliminary data suggests that ECM proteins undergo turnover at MTJs \textit{in vivo} but how is ECM turnover regulated during morphogenesis? We believe that it is probably controlled by the same regulators as Integrin turnover, such as mechanical force and Integrin activation. I propose performing FRAP analysis on a diverse array of fly ECM components under chemical activation and mechanical force modulation. Along with the turnover analysis, a mathematical model that describes ECM kinetics should be developed.

4.7 Summary

This research demonstrates that the strict control of turnover in cell-ECM adhesion, due to mechanical force and Integrin activation, is essential for transitioning from tissue morphogenesis to maintenance.
REFERENCES


Shattil, S.J., C. Kim, and M.H. Ginsberg. 2010. The final steps of integrin activation: the end game. Nat Rev Mol Cell Biol. 11:288-300.


APPENDIX: Supplemental materials and methods

Mathematical model for Talin and other IAC proteins

Mathematical modeling

We fit the FRAP experiments to the following mathematical model (Figure 3.10). Fluorescent Talin exists in three compartments in the model: free Talin (P), Talin as part of an internalized Integrin complex (B_c), and Talin bound at the membrane, either as part of an adhesion complex or bound to Integrin (M). We assume that the observed fluorescence recovery dynamics are determined by two independent mechanisms related to Integrin localization. First, Talin binds to membrane Integrins at rate \( k_{on} \) and unbinds at rate \( k_{off} \). Second, we consider the formation and disassembly of membrane adhesion complexes containing fluorescent Talin. These are assembled and disassembled at rate \( k_{asm} \) and \( k_{dis} \) respectively. For simplicity we assume that Talin associated with adhesion complexes remains in the context of such complexes whether internalized or at the membrane. We neglect diffusive transport in the membrane since it was previously shown to be unimportant on the timescale of the experiments we fit here (~300 seconds) (Yuan et al., 2010). As in previous work (Pines et al., 2012), we also assume that the FRAP protocol does not disrupt chemical equilibrium of the reactants. Finally, we note that the fluorescently labeled Talin may undergo background photobleaching. We therefore correct the FRAP data by including a photobleaching parameter \( \delta \) in the equation for the membrane
concentration $M$. We assume that photobleaching will only affect fluorescent proteins in the membrane.

Under these assumptions, the dynamics can be described with the following system of equations,

$$
\frac{dM}{dt} = -(k_{off} + k_{dis} + \delta)M + k_{on}P + k_{asm}B_c
$$

$$
\frac{dP}{dt} = -k_{on}P + k_{off}M
$$

$$
\frac{dB_c}{dt} = k_{dis}M - k_{asm}B_c
$$

with initial conditions

$$
M(0) = \begin{cases} 
0 & \text{recovery region} \\
M_{eq} & \text{reference region} 
\end{cases}, \quad P(0) = P_{eq}, \quad B_c(0) = (B_c)_{eq}.
$$

By assuming that the system is at chemical equilibrium and that there is no photobleaching prior to the start of the experiment, we obtain the pre-bleaching equilibrium conditions:

$$
\frac{dP}{dt} = -k_{on}P + k_{off}M = 0 \quad \Rightarrow \quad \frac{P_{eq}}{M_{eq}} = \frac{k_{off}}{k_{on}}
$$

$$
\frac{dB_c}{dt} = k_{dis}M - k_{asm}B_c = 0 \quad \Rightarrow \quad \frac{(B_c)_{eq}}{M_{eq}} = \frac{k_{dis}}{k_{asm}}
$$

We then normalize the system by $M_{eq}$ to be able to compare all the experiments, and define:

$$
m = \frac{M}{M_{eq}}, \quad p = \frac{P}{M_{eq}}, \quad c = \frac{B_c}{M_{eq}}.$$
Then the normalized fluorescence recovery is described by system (2),

\[
\frac{dm}{dt} = -(k_{\text{off}} + k_{\text{dis}} + \delta)m + k_{\text{on}}p + k_{\text{asm}}c \\
\frac{dp}{dt} = -k_{\text{on}}p + k_{\text{off}}m \\
\frac{dc}{dt} = k_{\text{dis}}m - k_{\text{asm}}c
\]

with initial conditions

\[
m(0) = \begin{cases} 0 & \text{recovery region} \\ 1 & \text{reference region} \end{cases}, \quad p(0) = \frac{P_{\text{eq}}}{M_{\text{eq}}} = \frac{k_{\text{off}}}{k_{\text{on}}}, \quad B_c(0) = \frac{(B_c)_{\text{eq}}}{M_{\text{eq}}} = \frac{k_{\text{dis}}}{k_{\text{asm}}}.
\]

The system (2) is linear and \( m \) corresponds to the normalized recovery data, while \( p \) and \( c \) are not observable from the FRAP data. To find the best estimates of the parameters, we solve the system numerically and minimize the sum of square residuals between the solution of \( m \) and the data simultaneously as described in Pines et al., 2012.

**Parameter estimation and bootstrap confidence intervals**

To find the confidence intervals on the parameter values, we synthetically created new sample sets by bootstrapping. Bootstrap data was generated as previously described in Pines et al., 2012. This distribution is an approximation to the real distribution of the estimates and we use it to generate confidence intervals for each parameter value.

However, the missing information about the cytosolic variables \( p \) and \( c \) creates an inherent symmetry in the system. If we exchange \( k_{\text{dis}} \leftrightarrow k_{\text{off}} \) and \( k_{\text{asm}} \leftrightarrow k_{\text{on}} \) we will end with an identical equation for \( m \) but with opposite interpretations of \( p \) and \( c \).
Unsurprisingly, the resulting bootstrap distribution was found to be bi-modal for all our
data sets.

In order to differentiate disassembly from disassociation and assembly from binding,
we experimentally used an Integrin endocytosis inhibitor called dynasore. We assume that
by using dynasore to inhibit endocytosis we will decrease the disassembly rate. We also
assume that this inhibition does not affect any of the other interactions in the model.
Different concentrations of dynasore were needed to inhibit the endocytosis and modify the
disassembly for the different Talin mutants. To use dynasore, a solvent (DMSO) is needed.
We found no statistically significant difference between a sample with the corresponding
concentration of DMSO only and a sample with any modification. Thus we use solvent
alone as our control sample.

We compared samples with dynasore to control samples by assuming only the
disassembly rate differs between them. Call the disassembly rates $k_{\text{disC}}$ for the control data,
and $k_{\text{dis}}$ for the dynasore samples. We then fit the control and dynasore data simultaneously
to model (2) with $k_{\text{on}}$, $k_{\text{off}}$, $k_{\text{asm}}$ and $\delta$ held the same for both data sets. $k_{\text{disC}}$, $k_{\text{dis}}$ different.
We repeated this simultaneous fitting for the bootstrap samples. With this “double fitting”
procedure, the endocytosis inhibition data allows us to break the symmetry inherent in the
basic model and distinguish the estimated distributions for each of the parameters of the
model.

For each Talin mutant in a WT (non-mechanosensitive) background, we created
dynasore and control data sets and performed double fitting to estimate the parameter
distributions. Then, under the assumption that the true parameters for the control data
should be close to those obtained by using the double fitting approach, we choose appropriately from the bimodal single fit distribution to get the confidence intervals for the parameters of each Talin mutation. The dynasore data set was only used to compute the double fit.

In the mechanosensitive backgrounds, we could not use dynasore since the drug protocol along with the temperature change required to activate mechanosensitivity was found to compromise the health of the embryo or larvae. To overcome this limitation we make the assumption that there shouldn't be a great difference between the mechanosensor data and the control data parameter estimates. Therefore, we compare the parameter distributions of mechanosensor mutants for each of the Talin mutants with their corresponding control double fit distribution, and select the parameter distribution that is most similar to the parameter distribution from double fitting of the control.

Mathematical model for Integrin

Mathematical modelling

FRAP recovery curves were fitted to a two-compartment mathematical model similar to the model described in previous work (Pines et al., 2012). In this version of the model, Integrins exist in two distinct compartments – at the membrane (with concentration $M$) and within the cell (vesicular concentration $V$). Integrins in the membrane are assumed to undergo internalization (via endocytosis) at a fixed rate $k_{\text{endo}}$ and vesicles are assumed to be externalized (via exocytosis) at a fixed rate $k_{\text{exo}}$. We neglect diffusive transport in the membrane since it was previously shown to be unimportant on the timescale of the
experiments we fit here (~300 seconds) (Yuan et al., 2010). We applied the same protocol to obtain the FRAP recovery curves as in Hakonardottir et al., 2015 to improve the data collection and the robustness of model parameter fitting. We add a correction for the background photobleaching of fluorescently labelled proteins in the membrane compartment, with fixed rate parameter \( \delta \).

Assuming that the FRAP protocol does not disrupt the chemical equilibrium of the Integrin system, we describe the dynamics of Integrin with the following system of equations,

\[
\begin{align*}
\frac{dM}{dt} &= -(k_{endo} + \delta)M + k_{exo} V \\
\frac{dV}{dt} &= k_{endo} M - k_{exo} V
\end{align*}
\]

with initial conditions

\[
M(0) = \begin{cases} 0 & \text{recovery region} \\ M_{eq} & \text{region reference} \end{cases}, \quad V(0) = V_{eq}.
\]

As in previous work (Pines et al., 2012), we assume the system is at chemical equilibrium with no photobleaching prior to the start of the experiment. This allows us to assume the equilibrium condition at \( t=0 \):

\[
\frac{dM}{dt} = -k_{endo} M + k_{exo} V = 0 \quad \Rightarrow \quad \frac{M_{eq}}{V_{eq}} = \frac{k_{exo}}{k_{endo}}
\]
In order to compare different replicates of the FRAP experiments, we normalize, introducing the variables \( m = \frac{M}{M_{eq}} \), \( v = \frac{V}{M_{eq}} \). The normalized system of equations is thus

\[
\frac{dm}{dt} = -(k_{endo} + \delta)m + k_{exo}v , \quad \frac{dv}{dt} = -k_{exo}v + k_{endo}m \quad (2)
\]

with initial conditions

\[
m(0) = \begin{cases} 0 & \text{recovery region} \\ 1 & \text{reference region} \end{cases} , \quad v(0) = \frac{k_{endo}}{k_{exo}} .
\]

Here \( m \) represents the normalized labelled concentration of Integrin in the membrane. We assume that this quantity is proportional to the measured fluorescence. On the other hand \( v \) is not observable from the FRAP data. We use the analytic solution for \( m \) to find the best estimates of the parameters. The solution was found using usual techniques of differential equations and it is given by:

\[
m(t) = \begin{cases} c(e^{\lambda_+ t} - e^{\lambda_- t}) \\ c_+ e^{\lambda_+ t} + c_- e^{\lambda_- t} \end{cases}
\]

where,

\[
\lambda_\pm = \frac{k \pm \sqrt{k^2 + 4\delta k_{exo}}}{2} , \quad k = k_{exo} + k_{endo} + \delta , \quad c = \frac{k_{endo}}{\lambda_+ - \lambda_-} , \quad c_+ = \frac{\lambda_+ + \delta}{\lambda_+ - \lambda_-} , \quad c_- = 1 - c_+ .
\]
**Parameter estimation and bootstrap confidence intervals**

During the imaging acquisition, 75 frames were taken prior to the bleaching event and saved as a reference set to assist in estimating the background photobleaching rate. To find the best estimates of the parameters, we minimized the sum of square residuals between the analytic solution for $m$ and the observed fluorescence data. In order to achieve robust and consistent fits across the data, we found it was advantageous to weight the residuals for the reference region with weight 1/10 relative to the recovery region. To do the fitting we used a nonlinear least-square minimization algorithm using custom code written in Python as previous described (Pines et al., 2012). To find the confidence intervals on the parameter values, we synthetically created new samples using the bootstrapping technique previously described (Pines et al., 2012).

**Mobile fraction**

As described in Hakonardottir et al., 2015, the mobile fraction was calculated by fitting the exponential model typically used for FRAP data and ignoring background photobleaching. This was done independently of the two-compartment model fitting, providing a simple estimate of the fluorescence recovery fraction.

$$f(t) = f_{\text{max}} \left( 1 - e^{-\frac{t}{\tau}} \right)$$

The exponential model was fitted to each replicate individually. The mobile fraction for the complete sample was then computed as the average of the obtained estimates for $f_{\text{max}}$. 