ANALYSIS OF INTEGRIN TURNOVER IN DROSOPHILA MYOTENDINOUS JUNCTIONS

by

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ABSTRACT

Integrins are essential mediators of cell-ECM adhesion and they are, therefore, important to animal viability. Integrin-mediated transient (short-term) cell adhesion underlies dynamic processes such as cell migration while integrin-mediated stable (long-term) cell adhesion is essential for maintaining tissue architecture. Ongoing adhesion complex turnover is essential for transient cell adhesion, but it is unknown whether turnover is also required for maintenance of long-term adhesion. Fluorescence Recovery After Photobleaching (FRAP) was used to analyze the dynamics of the Integrin Adhesion Complex (IAC) in a model for long-term cell-ECM adhesion, Myotendinous Junctions (MTJs), in fly embryos and larvae. It was found that the IAC undergoes turnover in the MTJs and that this process is mediated by clathrin-dependent endocytosis but not lateral diffusion. Moreover, the small GTPase Rab5 can regulate the proportion of IAC components that undergo turnover and altering Rab5 activity weakened MTJs such that it leads to muscle attachment defects. In addition, growth of the MTJs was concomitant with a decrease in the proportion of IAC components undergoing turnover and it is possible that this growth-dependent decrease is regulated by the mechanical tension exerted on MTJs by muscle contraction. Experiments using mutations that result in increased mechanical tension exhibited lower IAC turnover. In contrast, mutations that lower mechanical tension exhibited higher IAC turnover with the exception of integrins. Therefore, we propose that IAC turnover is regulated during development by mechanical tension in long-term cell-ECM adhesions to allow normal tissue growth and maintenance.

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PREFACE

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This paper is incorporated with modifications into Chapter 2, Chapter 3.1-3.4, and Chapter 4.1-4.4.

Work presented in Yuan et al., 2010 was designed by Dr. Guy Tanentzapf and Lin Yuan. Lin Yuan performed all the fly genetics as well as all the cell biology experiments as described in Chapter 2.2-2.5. Michael J. Fairchild performed all the molecular biology as described in Chapter 2.1. Dr. Guy Tanentzapf and Alexander D. Perkins helped with the initial set up of the FRAP experiments. Research data was analyzed solely by Lin Yuan. The manuscript was prepared by Dr. Guy Tanentzapf and Lin Yuan.

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DEDICATION

For my family

CHAPTER 1: INTRODUCTION

1.1. <u>Integrins as adhesion mediators</u>

1.1.1. Integrins

Integrins are a major family of cell adhesion receptors conserved in all metazoans. They are heterodimeric proteins and each heterodimer is made up of non-covalently associated α and β subunits. Both α and β subunits are single-pass type I transmembrane proteins that each contain a large extracellular domain, a single transmembrane domain, and a small cytoplasmic tail (Springer and Wang, 2004; Arnaout et al., 2005; Fig. 1). So far, 18 α and 8 β subunits have been characterized in the mammalian genome that dimerize in 24 known combinations with cell-type-specific expression patterns (Hynes, 2002).

Although a small number of integrin αβ dimers can mediate cell-cell adhesion by binding to membrane bound receptors located on an adjacent cells (for example, E-cadherin, ICAM, VACM-1, MadCAM-1, and PECAM-1), the majority of integrin heterodimers mediate adhesion to the extracellular matrix (ECM) (Humphries et al., 2006). Mammalian integrins can bind to a variety of ECM ligands including but not restricted to fibronectin, vitronectin, collagen, and laminin (Humphries et al., 2006). Different integrin heterodimers have specificity for different ECM ligands although there is overlap. Some integrin heterodimers have affinity for multiple ECM ligands and the same ECM ligand can bind to a few different integrin heterodimers. Integrin-ECM interaction is often

Figure 1 – **Domain structures of integrin heterodimers**: integrins are heterodimers made up by an α (red) and a β (blue) subunits. Both α and β subunits contain large extracellular domains, single-pass transmembrane domains, and short cytoplasmic tails. Integrin heterodimers can adopt a number of conformations ranging from an inactive state (left) to an activated state (right) (Reprinted by permission from Macmillan Publishers Ltd: [NATURE REVIEWS MOLECULAR CELL BIOLOGY] Shattil et al., 2010, copyright (2010)).

β-Propeller

βA-domain

Hybrid domain

Thigh domain

αIIb integrin

PSI domain

β3 integrin

Calf 1 domain

Calf 2 domain

β-Tail domain

Transmembrane domain

Cytoplasmic domain

Figure 1 - Domain structures of integrin heterodimers

mediated by small, well-defined recognition sequences, such as the well-characterized Arginine-Glycine-Aspartate (RGD) motif, a conserved feature among many integrin ligands including fibronectin, vitronectin, and fibrinogen in vertebrates and Tiggrin in *Drosophila*. Besides RGD motifs, integrin can also interact with LDV motif in alternatively spliced regions of fibronectin and GFOGER motif in a specific conformation of collagen. Moreover, some ECM molecules like laminin, contain the RGD motif only in some splice variants and not in others, evidencing that ECM binding is still not completely understood (Humphries et al., 2006; Barczyk et al., 2009).

Given the complexity and diversity of integrin-mediated adhesion in vertebrate systems, the less genetically redundant invertebrate model organisms have proven useful for elucidating the roles integrins play in different biological processes. Toward this end, integrins have been extensively characterized in the fruit fly, *Drosophila melanogaster*. Five α (αPS1-5) and two β subunits (βPS and βν) have been discovered. Historically *Drosophila* integrins were named "PS" for their position specific expression pattern in the wing disc epithelia, where different α subunits are restricted either to the dorsal side (PS1) or the ventral side (PS2) (Brower et al., 1984). A number of the known ECM ligands of integrins including laminin, collagen, and thrombospondin are conserved between vertebrates and *Drosophila*, whereas others are not. For example, fibronectin is specific to vertebrates and Tiggrin is specific to *Drosophila* (Brown et al., 2000; Chanana et al., 2007). Tiggrin contains one RGD motif and is found in the tendon cell matrix where muscles attach (Bunch et al., 1998). Flies that lack Tiggrin show weak integrin-

like phenotypes (see integrin null phenotypes in section 1.3) that suggest a role in integrin-dependent muscle attachment (Bunch et al., 1998).

1.1.2. The Integrin Adhesion Complex (IAC)

1.1.2.1.   Overview

Integrins are capable of mediating strong cell-ECM adhesion when simultaneously bound to ECM-ligands and to the cytoskeleton. Most integrins are coupled to actin through the Integrin Adhesion Complex (IAC), a large complex of cytoplasmic proteins (Liu et al., 2000; Delon and Brown, 2009). One notable exception, in vertebrates, is α6β4 integrin, which aggregates to make up hemidesmosomes by linking the ECM to intermediate filaments via the scaffolding protein plectin (Rezniczek et al., 1998; Geerts et al., 1999; Homan et al., 2002). IAC components that can link integrins to actin filaments include talin, tensin, vinculin, α-actinin, filamin, melusin, skelemin and parvin (Burridge and Connell, 1983; Wilkins et al., 1986; Le Clainche et al., 2010; Wehland et al., 1979; Heggeness et al., 1977; Brancaccio et al., 1999; Prince, 1987; Nikolopoulos et al., 2000). Other important IAC proteins include Integrin-Linked Kinase (ILK), Focal Adhesion Kinase (FAK), Src family kinases, paxillin, kindlin, PINCH, Wech, and others (Hannigan et al., 1996; Schaller et al., 1992; Golden et al., 1986; Rendu et al., 1989; Huang et al., 1991; Pestina et al., 1997; Turner et al., 1990; Kloeker et al., 2004; Tu et al., 1999; Löer et al., 2008; Zaidel-Bar et al., 2007). Here, however, I will focus on discussing three core structural proteins in the IAC: talin, ILK and tensin, because they were used as IAC markers in studies described in this thesis.

1.1.2.2.   *Talin*

Talin is a large (~270kDa) cytoskeletal linker protein that was originally named after its cellular expression pattern as it was found at the end ("ankle" [1]) of stress fibers in adhesion plaques (Burridge and Connell, 1983). Talin exists in elongated (60 nm) flexible antiparallel homodimers (Gingras et al., 2008). It can be cleaved by the protease calpain into two parts: a larger C-terminal Rod domain (220kDa) and a smaller globular N-terminal head domain (47kDa) (Beckerle et al., 1987). The head domain contains a FERM (protein 4.1, ezrin, radixin, moesin) domain (subdivided into F1, F2, and F3), which is proceeded by the F0 domain. (Critchley, 2008; Fig. 2). The rod domain of talin is made up of 62 amphipathic α-helices, and talins in insects contain a poorly conserved and unstructured tail at the C-terminus (Critchley, 2008; Brown et al., 2002; Fig. 2).

Talin has two main functions: it acts as a scaffolding molecule and as a regulator of integrin binding to the ECM. Additionally, talin indirectly regulates cadherin expression in an integrin independent manner in the follicular epithelia of *Drosophila* (Bécam et al., 2005). Talin acts as a scaffolding protein for the assembly of a large intracellular Integrin Adhesion Complex (IAC), which links integrins to the actin cytoskeleton at cell-ECM attachments (Priddle et al., 1998; Tanentzapf and Brown, 2006; Appendix A). This link is

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[1] The word "talin" was derived from the Latin word for ankle, talus.

Figure 2 – **Schematic representation of talin domains**: The N-terminal talin head contains a F0 subdomain and a FERM domain comprising F1, F2, and F3 subdomains which are shown as blue circles in the figure. The C-terminal talin rod contains 62 amphipathic α-helices, and each pair of helices is represented as a blue oval in the diagram. In insects, talin also has an unstructured C-terminal tail following the rod domain. The numbering of primary proteins and helices are referred to the vertebrate talin1. Two integrin-binding sites have been found in talins: one in the F3 and one in the rod. There are three known actin-binding sites as well as several vinculin-binding sites in talins. Other proteins such as PIP kinase and layilin can also interact with talins. Finally, the F3 subdomain can interact with the rod domain to form an autoinhibited structure that prevents F3 from binding to integrins.

F3-talin rod autoinhibitory
intramolecular interactions

N--

F0 F1 F2 F3

PIP kinase, layilin

Calpain

--C

Unstructured tail
(insects only)

β-integrin binding sites
(F3, 1974-2293)

Vinculin binding sites (helices 4, 6, 9,
11, 12, 27, 33, 36, 46, 50, 58)

Actin binding sites (F2F3,
951-1327, 2300-2541)
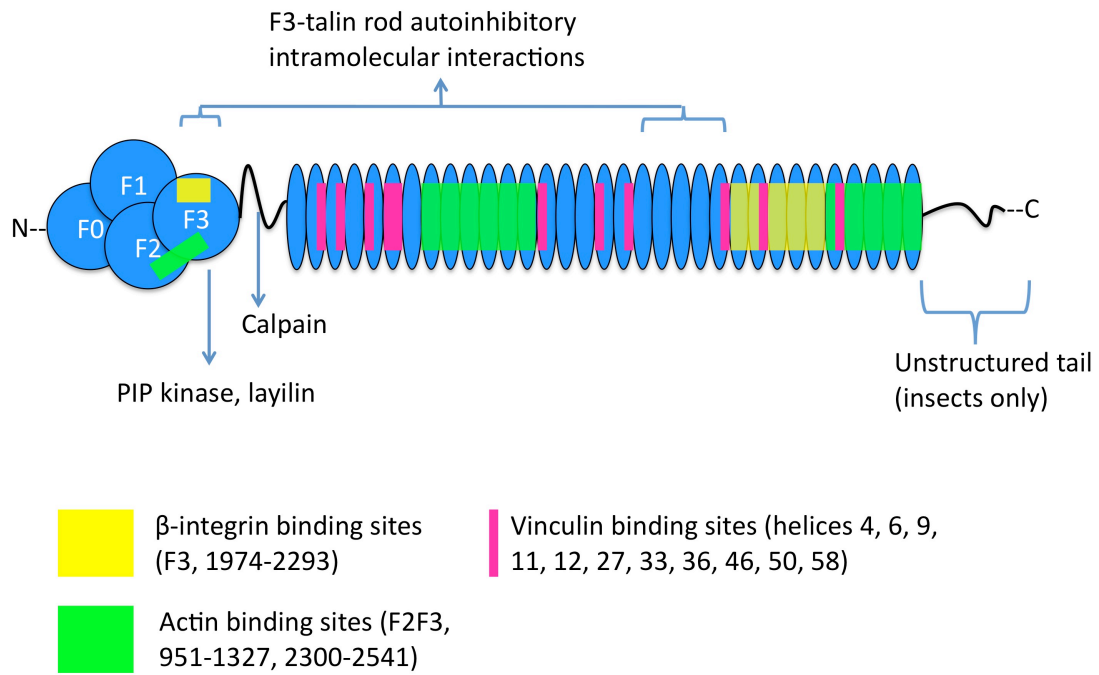
Figure 2 - Schematic representation of talin domains

achieved by binding to the cytoplasmic tail of β integrin directly and to the actin cytoskeleton either directly via several actin-binding domains throughout its length or indirectly through vinculin and other actin binding proteins (Critchley and Gingras, 2008; Fig. 2; Appendix A). Talin contains a number of known protein interaction domains located both in the head and the rod domains that include: two Integrin Binding Sites (IBS), three Actin Binding Domains (ABD), several Vinculin Binding Sites (VBS), as well as binding domains for other IAC components such as FAK, TES, layilin, and the PIP kinase PtdInsPKI γ-90 (Horwitz et al., 1986; Tremuth et al., 2004; Hemmings et al., 1996; Lee et al., 1992; Chen et al., 1995; Coutts et al., 2003; Borowsky et al., 1998; De Paolo et al., 2003; Critchley and Gingras, 2008; Fig. 2). Moreover, talin is an essential regulator of integrin affinity for ECM ligands ("inside-out activation", detailed below in section 1.2.2) and is therefore a regulator of adhesion dynamics making it important for a wide range of biological processes that rely on integrin-based adhesion (Nayal et al., 2004; Shattil et al., 2010).

Vertebrates have two talin genes: *Tln1* and *Tln2*. The disruption of *Tln1* in mice gives rise to lethality due to defective gastrulation during early embryonic development (Monkley et al., 2000). Moreover, mice that lack both talin1 and 2 exhibit defects in myoblast fusion, sacromere assembly, and maintenance of myotendinous junctions resembling defects in mouse muscles lacking β1 integrin (Conti et al., 2009). In *Drosophila*, there is only one talin homologue, which is encoded by the *rhea* gene (Prout et al., 1997). Mutations in *rhea* lead to embryonic lethality and show striking resemblance to the integrin null mutant phenotypes in the fly including defects in germ-band retraction,

dorsal closure, muscle attachments, and wing adhesion (Brown et al., 2002; Tanentzapf and Brown, 2006).

1.1.2.3.   Integrin-Linked Kinase (ILK)

The Integrin Linked Kinase (ILK) gene was first identified in a yeast-two-hybrid screen designed to uncover binding partners for the β1 integrin cytoplasmic tail (Hannigan et al., 1996). ILK is a 59kDa protein that contains three types of conserved motifs: four ankyrin-like repeats at the N-terminus, a putative pleckstrin homology (PH) domain, and a serine/threonin kinase domain at the C-terminus (Hannigan et al., 1996; Delcommenne et al., 1998). Apart from its ability to bind to β1 and β3 integrins in mammals, ILK is also central to the ternary complex of ILK, PINCH, and parvin (IPP complex), which has shown to be an essential link between integrin and actin filaments (Legate et al., 2006; Wickström et al., 2010; Zervas et al., 2001). Recent work has argued that the putative kinase activity, for which ILK is named after, is not biologically relevant since the domain originally identified to be a kinase domain is inactive (Lange et al., 2009; Wickström et al., 2010). Nonetheless, ILK has essential roles in IAC assembly and maintenance by targeting IPP complex to integrin adhesion sites during development of mice, flies, worms and zebrafish (Lange et al., 2009; Zervas et al., 2001; Mackinnon et al., 2002; Postel et al., 2008). In *Drosophila*, loss of ILK leads to defects that resemble but are not as severe as the integrin loss of function phenotypes (Zervas et al., 2001). In addition, in the nematode *C. elegans*, loss of ILK gives rise to a Pat phenotype (Paralysed Arrested elongation at Twofold) that is characteristic of mutations that abolish integrin function in *C. elegans* (Mackinnon et al., 2002). Recent cell culture studies also suggest

that ILK has important roles in the organization of the microtubule network and mitotic spindle orientation, although this has not yet been shown to be the case in invertebrate model systems (Dobreva et al., 2008; Fielding et al., 2008).

1.1.2.4.   *Tensin*

Tensin, another important component of the IAC, was originally named because of its putative function in maintaining tension in the microfilaments (Davis et al., 1991;Wilkins et al., 1986). Tensin is a protein with approximate molecular weight of 220 kDa and contains several conserved domains that can bind to structural or signaling proteins (Lo, 2004). Tensin has multiple actin binding sites that allow it to cap actin barbed ends, cross-link actin filaments, and promote actin aggregation (Lo et al., 1994). Tensin also contains an Src Homology 2 (SH2) domain, which is capable of binding tyrosine-phosphorylated proteins like PI3K, p130Cas, and FAK (Davis et al., 1991; Lo, 2004). Moreover, tensin's Phospho-Tyrosine Binding (PTB) domain can interact with Asparagine-x-x-Tyrosine (NxxY) motifs on β integrin tails (Calderwood et al., 2003; Torgler et al., 2004). Tensin null mice and tensin null flies are reported to be viable and generally healthy. However, Tensin knockout mice exhibit defects in renal function, skeletal muscle regeneration, and fibroblast migration; and fly tensin null mutants have weakened integrin-mediated adhesion in the wing showing that tensin is an important component of the IAC (Lo, 2004; Torgler et al., 2004).

1.1.3. Types of integrin-mediated adhesions

Integrins and the IAC are capable of mediating both short-term and long-term cell adhesions. Long-term adhesions have the lifetime of hours, days, or even longer. They are important in fully formed tissues that are maintained throughout the organism's lifetime. The *Drosophila* Myotendinous Junction (MTJ) is a well-established model system to study integrin mediated stable and long-term adhesions. In *Drosophila*, both somatic muscle cells and tendon cells contain integrins, αPS2βPS in muscle cells and αPS1βPS in tendon cells, which allow them to attach to the ECM. The structure formed by muscle cells, tendon cells, and the ECM is termed the Myotendinous Junction (MTJ) (Fig. 3). Once MTJs are formed in late embryonic development, they are maintained throughout larval stages (5 days in room temperature) until pupation. After the second round of morphogenesis in pupal stages, the reassembled MTJs are maintained up to 6 weeks in adults.

A well-studied model for short-term integrin-mediated adhesion is nascent adhesions (or focal contacts) in migrating cells. Nascent adhesions are small, less than 1um in width, and are not linked to actin stress fibers (Zamir and Geiger, 2001). Upon formation, nascent adhesions can undergo turnover (see 1.4), which enables cell migration, or they can mature into more stable focal adhesions (Zamir and Geiger, 2001). Focal adhesions (or focal complexes) are oval shaped protein complexes that connect to the actin stress fibers (Zamir and Geiger, 2001). Although focal adhesions are larger and more stable than nascent adhesions, they are still smaller and significantly more dynamic than stable

Figure 3 – **Schematic diagram of Myotendinous Junctions (MTJs) in *Drosophila*:** MTJs are the structures in *Drosophila* where tendon cells and somatic muscle cells attach to the ECM. Both somatic muscle cells and tendon cells express surface integrins, αPS2βPS in muscle cells and αPS1βPS in tendon cells, which allow them to attach to the ECM. αPS2βPS is also connected to the actin filaments in myofibrils through the IAC, while αPS1βPS is connected to the cortical actin cytoskeleton.

Epidermis

Tendon

Muscle

αPS2βPS	ECM	Myofibril	
αPS1βPS	IAC	Cortical Actin	
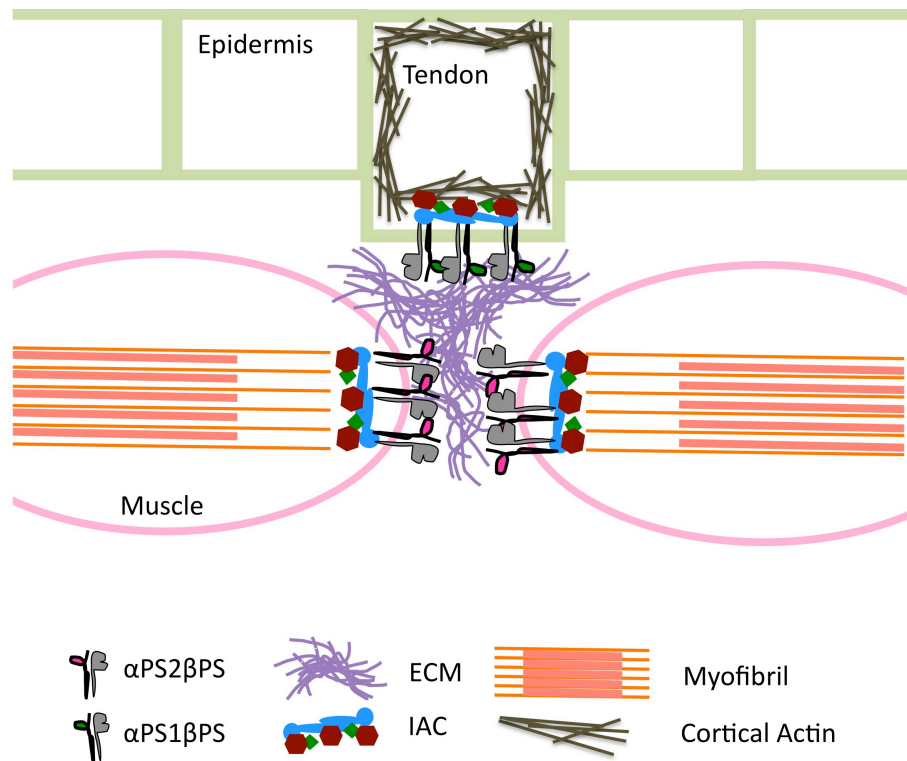
Figure 3 - Schematic diagram of Myotendinous Junctions (MTJs) in *Drosophila*

and long-term adhesions, like *Drosophila* MTJs, as their lifetime is in the order of minutes or hours (Smilenov et al., 1999).

1.2. Integrins as signaling receptors

1.2.1. Overview

Starting in the 1980s, evidence of integrin's involvement in regulating gene expression and cell differentiation started to accumulate but it was not until the observation of integrin-mediated regulation of tyrosine kinases in platelets and the subsequent discovery of FAK that confirmed the dual function of integrins as both adhesion and signaling receptors (Rohrschneider, 1980; Ferrell and Martin, 1989; Golden et al., 1990; Hanks et al., 1992; Schaller et al., 1992). Integrins can mediate bidirectional signaling; the binding of talin and/or other activating proteins to the cytoplasmic tail of integrins initiates "inside-out signaling" that can regulate cell adhesion, cell migration, and ECM assembly and remodeling, whereas the binding of ECM ligands to the extracellular domains of αβ heterodimer results in "outside-in signaling", which can control cell polarity, cell proliferation, cytoskeletal structure, and gene expression (Huttenlocher et al., 1996; Palecek et al., 1997; Wu et al., 1995; Harburger and Calderwood, 2009; Legate et al., 2009; Shattil et al., 2010). Although inside-out and the outside-in signaling have distinct biological consequences, they can indirectly feed into one another *in vivo*. For example, inside-out signaling can increase ligand binding, which will lead to increased outside-in signaling; conversely, outside-in signaling can generate signals that recruit or activate talin and other proteins that trigger inside-out signaling (Calderwood, 2004; Goksy et al.,

2008; Harburger and Calderwood, 2009). Recently, structural analysis of transmembrane domains of αIIbβ3 integrins has shed light on mechanisms enabling integrins to propagate signals bidirectionally (Lau et al., 2009). The signaling network involving integrins is incredibly complex. Thus far, 156 signaling, structural, and adaptor molecules mediating 690 interactions have been identified in the integrin signaling network: the "integrin adhesome" (Zaidel-Bar et al., 2007).

1.2.2. Inside-out signaling

Integrins can adopt a number of conformations characterized by different affinities for ECM ligands and the strength of integrin-mediated adhesion can be regulated by these conformational changes (Frelinger et al., 1990). Although there are a number of competing models about the precise nature of integrin regulation by conformational changes, it is generally thought that integrin dimers can have three major conformational states: inactive (low affinity for ECM ligands), primed (high affinity for ECM ligands) and ECM-ligand occupied (Askari et al., 2009). Binding of activating proteins like talin to the integrin cytoplasmic domain can lead to conformational changes in the integrin extracellular domain such that the ECM ligand binding affinity is increased; this corresponds to a change from an inactive to a primed conformation (Shattil et al., 2010). This process is called "inside-out activation", which is the first step of "inside-out signaling" (Shattil et al., 2010).

The interaction of the cytoplasmic tails of α and β integrin with intracellular adapter molecules is essential for IAC recruitment and inside-out activation (Calderwood, 2004; Tanentzapf et al., 2006). The cytoplasmic tail of the α integrin subunit is important for integrin activation, because deleting the conserved GFFKR motif in the membrane proximal regions of α integrin leads to constitutive activation (Calderwood, 2004). Mutagenesis and FRET analyses also demonstrated salt bridge associations between the membrane proximal regions of α and β tails block activation (Calderwood, 2004). However, the membrane distal regions of the known 18 mammalian α subunits are not well conserved complicating the research on cytoplasmic proteins that activate α tails (Calderwood, 2004; Shattil et al., 2010). Nevertheless, at least one activating α integrin binding protein has been characterized. This protein, RAPL (also known as NORE1 and RSSF5), is a Rap1-binding protein that associates with αL integrin and regulates αLβ2-integrin mediated adhesion in lymphocytes (Katagiri et al., 2003; Ebisuno et al., 2009). In contrast, the cytoplasmic domains of various β integrin subunits are highly conserved and the involvement of β integrin tails during inside-out activation is better understood. For example, deletion of the membrane proximal regions of the β tail results in constitutive activation, whereas deletion of membrane distal regions blocks activation (Calderwood, 2004). While the membrane proximal regions of the β tails seem to interact with α tails to stabilize inactive and low ligand affinity conformations, the conserved NxxY sequences in the β tail, and especially the membrane proximal Asparagine-Proline-x-Tyrosine (NPxY) motif, are important for binding of numerous cytoplasmic proteins and inducing conformational changes that lead to activation (Liu et al., 2000; Ulmer et al, 2001).

The binding of talin to integrin is thought to be the final common step in integrin activation (Tadokoro et al., 2003). Binding of talin to integrin through its N-terminal IBS sites, a PTB domain in the F3 subdomain of talin head, leads to activation of both mammalian and *Drosophila* integrins (Calderwood et al., 2002; Tanentzapf and Brown, 2006). Recent structural studies have provided insight on the mechanism of why talin, but not other proteins that contain PTB domains, is necessary for the activation of integrins. Specific ionic interactions between the talin F3 domain and the membrane-proximal helix of the β tail disrupt an integrin αβ salt bridge, thus inducing separation of the α and β cytoplasmic tails (Wegener et al., 2007; Lau et al., 2009). An electrostatic interaction between a group of positivity-charged lysine residues in the talin F2 domain and the negatively charged lipid face of the plasma membrane stabilizes and orients talin to promote the interaction between talin and β3-integrin (Anthis et al., 2009a). While talin binding is essential for inside-out activation, it is not known whether talin is by itself sufficient for integrin activation. Other proteins have been recently shown to cooperate with talins in activation: for example, kindlins can bind to the membrane distal NxxY motif on the β tail and help talin to fully activate integrins (Ma et al., 2008; Maitanez et al., 2008; Moser et al., 2008). However, *in vitro* studies also showed that vertebrate talin1 was by itself sufficient to activate αIIbβ3 in the presence of plasma membrane (Ye et al., 2010).

Following inside-out activation, the separation of the cytoplasmic tails of α and β integrin allows the recruitment with talin which then forms a platform for the recruitment of other IAC proteins like ILK, PINCH, and parvin, leading to the assembly of the IAC

(Legate et al., 2006). As talin recruitment by the cytoplasmic tail of β integrin is central for both integrin activation and subsequent IAC assembly, talin-integrin interactions are highly regulated. Src family kinases can mediate tyrosine phosphorylation of the membrane proximal NPxY motif, which blocks talin binding and hence regulates integrin activation (Kiema et al., 2006; Takala et al., 2008; Oxley et al., 2008; Anthis et al., 2009b). The integrin-talin interation can also be regulated by a mechanism involving reversible autoinhibition of talin. Talin can adopt a closed or autoinhibited conformation where it cannot interact with integrins (Fig. 2). This autoinihibition may be relieved by at least two different ways: proteolytic cleavage of the talin head domain by calpain2 (Beckerle et al., 1987; Crithley and Gingras, 2008) or, alternatively, by binding of the talin rod domain to PIP2 (PtdIns(4,5)P2) (Goksoy et al., 2008). Finally, inside-out activation of integrin might occur via a more indirect mechanism involving signaling events downstream signals received through other transmembrane receptors. For example, αIIbβ3 is activated through signaling of thrombin receptors in platelets: activation of thrombin-receptors turns on the downstream signaling molecule PKC, activated PKC employs Rap1, which in turn recruits talin and RIAM (Rap1 InterActing Molecule) to activate integrins (Shattil et al., 2010).

1.2.3. Outside-in signaling

When integrins bind to ECM ligands, they undergo conformational changes that are propagated from the extracellular domains via transmembrane domains to the intracellular domain. These conformational changes are eventually translated into signaling events inside the cell (Frelinger et al., 1990; Lau et al., 2009; Hynes, 2002;

Askari et al., 2009). Outside-in signaling is manifested by activation of certain kinases in the IAC such as FAK and Src family kinases (Ferrell and Martin, 1989; Golden et al., 1990; Schaller et al., 1992; Hanks et al., 1992). Moreover, integrin binding to their ECM ligands also gives rise to elevated concentration of lipid secondary messengers like PIP2 (PtdIns(4,5)P2) and PIP3 (PtdIns(3,45,)P3) (McNamee et al., 1993). Phosphoinositides can regulate integrin-mediated adhesion in a number of ways including: enhancing talin-integrin interactions, disabling the binding of α-actinin to actin, and modifying Akt derived signalling (Martel et al., 2001; Greenwood et al., 2000; Corgan et al., 2004; Vanhaesebroeck and Alessi, 2000).

In general, FAK is one of the central signaling scaffold proteins in integrin outside-in signaling (Zaidel-Bar et al., 2007). In particular, one of the cellular consequences of FAK activation is increasing adhesion complex turnover in cultured migrating cells, because FAK-deficient cells exhibit reduced motility and FAK overexpression results in enhanced migration (Ilic et al., 1995; Owen et al., 1999; Cary et al., 1996). Modulation of cell motility through FAK is highly complex and can be achieved through a plethora of downstream signaling events (Legate et al., 2009). Briefly, FAK promotes cell motility by two major ways. Firstly, FAK induces remodeling of actin filaments and the microtubule network by regulating Rho family GTPases, N-WASP, and α-actinin (Ren et al., 2000; Palazzo et al., 2004; Wu et al., 2004; Izaguirre et al., 2001; Mitra et al., 2005). Secondly, FAK can couple with Src and FAK-Src complex regulates adhesion dynamics by activating or recruiting other proteins that modulate integrin-mediated adhesion such as phosphatidylinositol lipids, calpain, and Matrix Metallo-Proteinases (MMPs) (Ling et

al., 2002; Carragher et al., 2003; Franco et al., 2004; Huang et al., 2009; Hauck et al., 2002; Takino et al., 2007).

Integrin-mediated signaling can intersect with other signaling pathways, especially signaling events involving Growth Factor Receptors (GFRs), through a process called "cross talk", which often leads to enhanced signaling designed to promote specific outcomes (Legate et al., 2009). Outside-in signaling initiated by ECM binding to integrin promotes cell growth and proliferation by the Extracellular signal-Regulated Kinase 2 (ERK2)/Mitogen-Activated Protein Kinase (MAPK) pathways that are downstream of GFRs (Schaepfer et al., 1994; Roovers et al., 1999; Legate et al., 2009). Notably, integrin-mediated cell attachment can activate Epidermal Growth Factor Receptors (EGFRs) in the absence of their ligands: Epidermal Growth Factors (EGFs) (Moro et al., 1998). Activated GFRs can also regulate cell adhesion and motility through modulating the kinase activity of FAK and c-Src (Sieg et al., 2000; Goi et al., 2000). An example of the sort of signaling cascade that links EGFRs to FAK and Src was recently described (Long et al., 2010). It was shown that PAK1 phosphorylates and recruites an isoform of SRC-3 to the plasma membrane, which leads to the phosphorylation of FAK and c-Src (Long et al., 2010). Another example of EGFR-integrin cross talk involves PI3K and Vav2, downstream components in the EGFR pathway that regulate actin polymerization and integrin recruitment through integrin-dependent activation of Rac (Marcou and Vuori, 2003; Kiosses et al. 2001).

1.2.4. Mechanotransduction

In addition to inside-out and outside-in signaling, the strength of the integrin-mediated cell-ECM adhesions is also regulated by mechanical tension generated both intrinsically, by actomyosin contraction and actin polymerization, and extrinsically, by increased rigidity of the ECM (Geiger et al., 2009). Owing to the complexity and bidirectional nature of integrin signaling, mechanotransduction is probably achieved through a combination of regulatory events downstream of integrins rather than through a single, structurally distinct module in integrin (Zaidel-Bar et al., 2007; Geiger et al., 2009). For example, mechanical forces have shown to independently affect a number of core structural and signaling components of the integrin-mediated adhesion, such as integrin, talin, fibronectin and p130Cas (Friedland et al., 2009; del Rio et al., 2009; Smith et al., 2007; Sawada et al., 2006).

Evidence has recently accumulated that integrins can form "catch bonds" with their ECM ligands. Catch bonds are a type of receptor-ligand bonds that are strengthened by tensile force as opposed to most receptor-ligand bonds that are weakened by tensile force (Thomas et al., 2008). In vitro studies on the leukocyte integrin receptor, αLβ2, demonstrate that the application of moderate force on αL induce conformational changes in the I domain of αL such that bring the αLβ2 heterodimer to a high affinity states (Astrof et al., 2006). Moreover, the application of a moderate force, on the order of 10-30 pN, was shown to prolong α5β1-ligand associations using atomic force microscopy (Kong et al., 2009). Molecular dynamic simulations also provide insights into the structural basis of the catch bonds between integrins and ECM ligands (Puklin-Faucher et

al., 2006; Puklin-Faucher et al., 2009). Ligand binding has shown to induce conformational changes in β integrins. For example, when β integrins are exposed to force, this can lead to the molecular changes that result in separation of the contact (or "hinge") site between the βA domain (also known as I-like or βI domain) and other internal sites in the extracellular domain of the β integrin molecule (Puklin-Faucher et al., 2009). These conformational changes have a stabilizing effect on the ligand-receptor bond by maintaining the active conformation of the βA domain (Puklin-Faucher et al., 2006; Puklin-Faucher et al., 2009). Recent *in vivo* studies demonstrated a catch bond between integrins and their ECM ligands directly by showing that both actomyosin contraction and ECM stiffness can regulate integrin-fibronectin bond strength in cultured cells (Friedland et al., 2009).

Another potential mechanism by which mechanical force regulates integrin-mediated function is by conferring conformational changes in other components of the integrin-mediated adhesions other than integrins. For example, sheer force can cause unfolding of the talin rod domain such that the typically hidden vinculin-binding sites are exposed (del Rio et al., 2009). Moreover, stretch-induced unfolding of the signaling protein p130Cas reveals a phosphorylation site for Src family kinases (Sawada et al., 2006). The subsequent phosphorylation of p130Cas by Src family kinases initiates further downstream signaling events (Sawada et al., 2006). Finally, ECM ligands like fibronection can also undergo conformational changes in response to mechanotransduction, which affect their receptor specificity and biochemical properties (Smith et al., 2007).

1.3. <u>Function of integrins in tissue development and maintenance</u>

1.3.1. Overview

Cell adhesion plays crucial roles during many processes in multicellular organisms including: embryonic development, tissue maintenance and repair, immune response, haemostasis, and cell division (Brown et al., 2000; Motell, 2008; Evans et al., 2009; Coller and Shattil, 2008; Pellinen et al., 2008). As development progresses from a single cell to an full grown organism, transient contacts that are helpful for cell migration and morphogenesis gradually mature into mature cellular junctions, which are larger, stronger, and more stable adhesion site that can withstand stronger mechanical force and maintain tissue integrity (Brown et al., 2000). While the cadherin family of cell adhesion molecules often mediates adhesion between cells of the same type, the integrin family of adhesion receptors is often responsible for connecting different cell types by linking them to the ECM (Brown et al., 2000). Notably, integrins must function dually as both signaling and adhesion receptors and are essential for embryogenesis and viability in animals as demonstrated by genetic studies in nematodes, insects, and mammalian model organisms. (Bokel and Brown, 2002). Here I will concentrate on roles that integrin-mediated adhesions play in tissue development and maintenance in mice and flies.

1.3.2. Integrins in mouse development

Vertebrates have 18 α subunits and 8 β subunits of integrins and knockout of different subunits in mice can give rise to a spectrum of phenotypes ranging from early embryonic

lethality in the case of β1-deficient mice to near wild type phenotype in the case of α1 integrin where mice appear grossly normal but have defects in wound healing (Sheppard, 2000). The different effects caused by knockout of different subunits may be due to the expression pattern of the different integrin subunits and their ability to form many kinds of heterodimers with many diverse roles. For example, β1 integrin is ubiquitously expressed and found in 12 out of 24 known αβ dimers whereas other integrin subunits such as α1 integrin show more restricted tissue specific expression and form few different heterodimers. However, this is not always the case, because, for example, loss of α2 integrin gives rise to early embryonic lethality even though it can only associate in a heterodimer with β1 integrin (Sheppard, 2000). This illustrates that different αβ dimers can have highly specialized, often essential, roles in development and/or tissue maintenance. Indeed, depletion of different β1 containing integrin heterodimers in mice results in unique phenotypes with very little overlap (Sheppard, 2000; Bouvard et al., 2001).

1.3.3. Integrins in *Drosophila* development and maintenance

It is sometimes hard to dissect integrin function in vertebrate development due to the large number of integrin heterodimers and the risk of functional redundancy between them (De Arcangelis and Georges-Labouesse, 2000; van der Flier and Sonnenberg, 2001). Most integrin subunits are evolutionarily conserved across the animal phyla and often this conservation also extends to the functional level (Bokel and Brown, 2002). It should be noted that some integrin subunits, like the α4, α9, and β4 integrins, are unique

to vertebrates as are certain functions of integrin adhesion such as platelet-mediated haemostasis (Bokel and Brown, 2002; Coller and Shattil, 2008). Nonetheless, the use of invertebrate model organisms, such as *Drosophila melanogaster*, which has a smaller number of potential integrin heterodimers, a shorter generation time, and powerful genetic tools, have proven very useful in elucidating the conserved basic functions of integrin-mediated adhesion (Bokel and Brown, 2002). Integrins play a large variety of roles during fly development including cell migrations of the visceral musculature, primordial midgut, trachea, haemocytes, and salivary glands (Bokel and Brown, 2002; Boube et al., 2001; Bradley et al., 2003). Moreover, integrins mediate a number of essential morphogenetic movements in the fly that are essential for embryogenesis including dorsal closure and germband retraction (Brown, 1994; Brown et al., 2000; Schöck and Perrimon, 2003).

Not only are integrins important during development, integrin-mediated adhesions are also important for tissue maintenance. In *Drosophila*, this is seen in the MTJs that are formed independently of integrin-mediated adhesion but required integrins to remain attached (Brown, 1993). Knockdown experiments of integrins in fully formed adult fly muscles, using RNAi, resulted in decreased life span as well as deteriorating muscle function and morphology (Perkins et al., 2010). Moreover, while integrin signaling is important for setting up the dorsal and ventral layers of epithelial cells that make up the adult fly wings, integrin mediated adhesions are necessary for maintaining connections between the two cell layers (Brabant et al., 1996; Brabant et al., 1998)**.** Furthermore, tracheal terminal branches are actively maintained by integrin-mediated adhesions (Levi

et al., 2006). Additionally, integrins are important for the maintenance of the of stem cell

niche in fly testes (Ellis and Tanentzpaf, 2010; Tanentzapf et al., 2007).

1.4. <u>Integrin turnover</u>

1.4.1. Mechanisms of transmembrane protein turnover

Fluorescent Recovery After Photobleaching (FRAP) was first used in the 70s to show

dynamics of transmembrane proteins (Edidin et al., 1976). The advent of tagging proteins

with non-invasive fluorescent proteins, such as GFPs, allowed the use of FRAP to

measure movements of molecules within or between defined cellular compartments in

living cells (Reits and Neefjes, 2001). FRAP involves photobleaching fluorescently

labeled proteins of interest within an area and recording the protein dynamic indicated by

fluorescence recovery over time (Reits and Neefjes, 2001). FRAP data is conventionally

plotted as a curve with time on the x-axis and fluorescence intensity on the y-axis and

these graphic representations are known as FRAP curves (Fig. 4). Mobile fraction, which

represents the amount of mobile proteins as a fraction of total amount of proteins, can be

obtained from FRAP analyses (Reits and Neefjes, 2001; Fig. 4).

Generally speaking, two models have been proposed in the field to explain the

fluorescence recovery of transmembrane proteins after photobleaching: lateral diffusion

across the plasma membrane and endo/exocytic cycle of protein recycling (Kusumi et al.,

1993; Sorkin and von Zastrow, 2009). Work in cell culture has shown using FRAP

Figure 4 – **Fluorescence Recovery After Photobleaching (FRAP):** (a) Fluorescently molecules that localize on the *Drosophila* embryonic MTJ inside the orange box are photobleached and the fluorescence intensity within the orange box is plotted against time in (b). Mobile fraction, which represent the amount of protein that show recovery as a fraction of total amount of proteins, can be obtained from FRAP analyses (b) and represented by a bar graph (c).
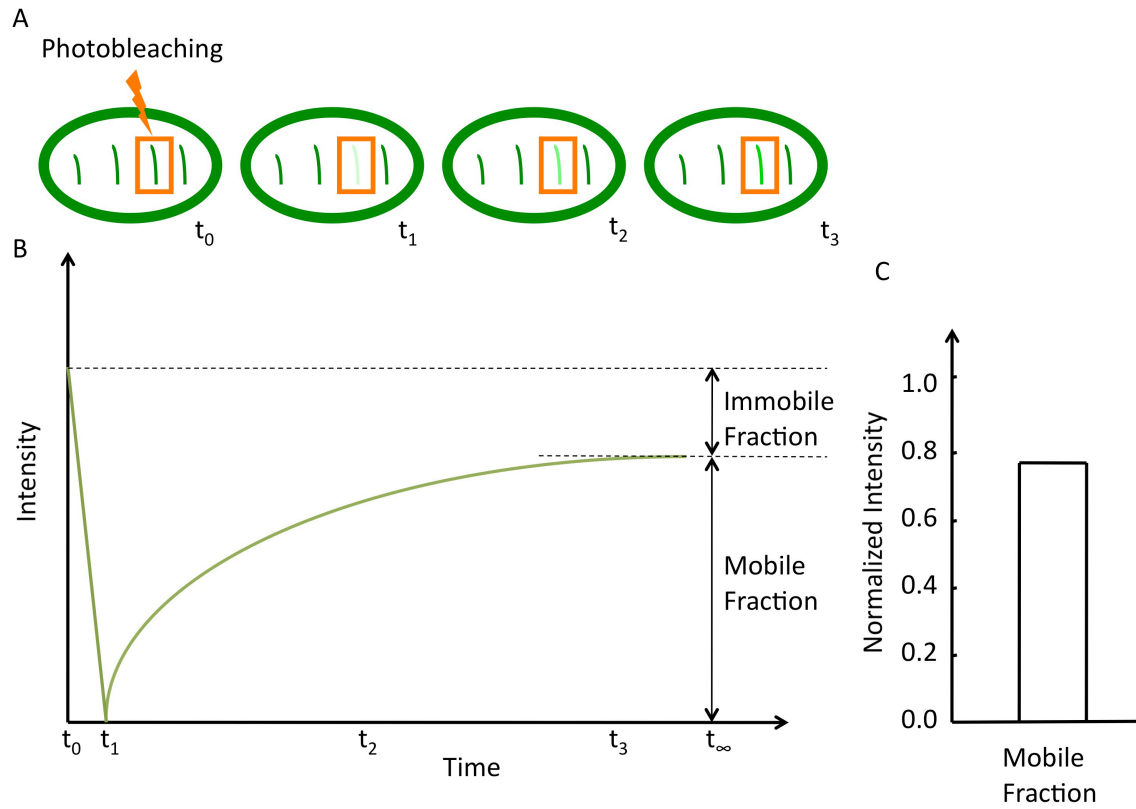
Figure 4 - Fluorescence Recovery After Photobleaching (FRAP)

analysis on fluorescently tagged integrins in migrating cells that integrins are highly dynamic at transient adhesion sites (Ballestrem et al., 2001). Evidence exists that both lateral diffusion and endocytic recycling play a role in the turnover of integrins (Fig. 5). One dynamic model of integrin based on cell culture research suggests that lateral diffusion is responsible for the integrin recovery observed through FRAP (Wehrle-Haller, 2007). This model infers that both integrin activation and clustering could influence integrin dynamics by slowing down the diffusion rate of integrins across the cell membrane (Wehrle-Haller, 2007). This is confirmed by the observation that forced integrin clustering could influence integrin dynamics; however, while no direct evidence showed integrin dynamics as a result of mere lateral diffusion (Cluzel et al., 2005; Wehrle-Haller, 2007). There is substantial support for the role of endocytic recycling in integrin turnover in migrating cells (Caswell et al., 2009; Fig. 5). Not only is endocytic recycling important for surface expression of integrins, it also plays key roles in focal adhesion assembly and disassembly in migrating cells (Ezratty et al., 2005; Ezratty et al., 2009).

1.4.2. Intracellular trafficking of integrins in cultured cells

Integrins were first observed to be endocytosed into the cytoplasm and subsequently recycled back onto the plasma membrane in cultured Chinease Hamster Ovary (CHO) cells (Bretscher, 1989; Bretscher, 1992). This dynamic property of integrins was proposed to enable cells to migrate by first disassembling focal adhesions at the trailing edge, and then transporting integrins within vesicles to the leading edge to form new focal adhesions (Brestscher, 1996). While no direct proof has been found showing

Figure 5 – **Schematic models of integrin dynamics at focal adhesions:** (a) in the lateral diffusion model, integrins are diffusing across the plasma membrane. Integrins originally in the focal adhesion can dissociate from the complex and diffuse away, while new integrins can diffuse to join an existing complex. (b) In the endocytic recycling model, endocytosis can lead to focal adhesion disassembly, which releases disassociated integrins. Disassociated integrins can be endocytosed into the cell and subsequently recycled back on to the membrane. These recycled integrins can be activated and recruited into pre-existing focal adhesions.

A. Lateral diffusion model

B. Endocytic recycling model

Integrin heterodimer

ECM

Integrin containing endosomes

Actin filaments

IAC
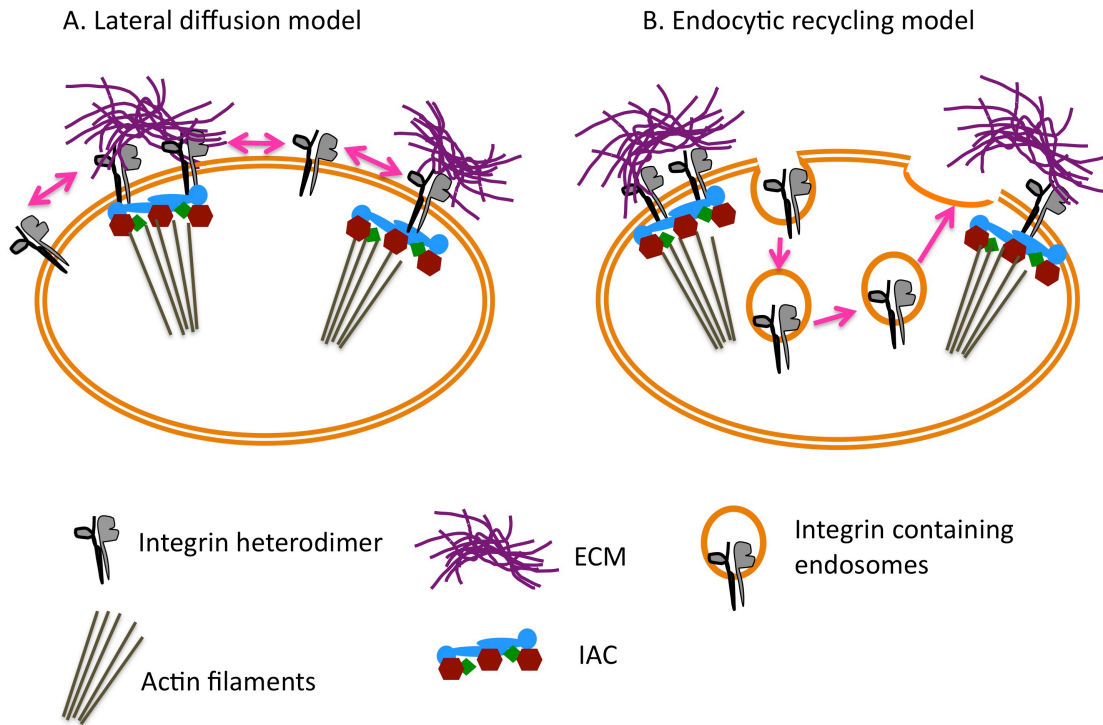
Figure 5 - Schematic models of integrin dynamics at focal adhesions

transport of integrins from the trailing edge to the leading edge during cell migration, evidence suggest that endocytic recycling of integrins regulates cell migration by controlling the integrin distribution in a restricted region of the cell (Caswell et al., 2007; Caswell et al., 2009).

Integrins can be internalized into the cell via clathrin-dependent or caveolin-dependent routes (Ezratty et al., 2009; Pellinen et al., 2006; Mosesson et al., 2008). Many integrin subunits and heterodimers were shown to undergo clathrin-mediated endocytosis. β integrins contain conserved NxxY motifs required to recruit other cell surface receptors to clathrin-coated structures. Mutations of the β1 integrin NxxY motifs lead to decreased clathrin-mediated β1 integrin endocytosis and reduced focal adhesion turnover in cultured cells (Pellinen et al., 2008). αvβ5 integrins were also visualized at clathrin-coated pits using electron microscopy (De Deyne et al., 1998). Recently, Dab2, a clathrin-associated endocytic adapter, was found to be responsible for internalizing inactive integrins on the cell surface and regulating cell migration (Teckchandani, et al., 2009). Moreover, clathrin is important for focal adhesion disassembly in migrating cells (Ezratty et al., 2009). In contrast, several integrins have been shown to associate with lipid rafts and may utilize lipid raft mediated protein trafficking. For example, αvβ3 integrin and α5β1 integrin associate with caveolin1 (Altankov and Grinnell, 1995). Down-regulation of Caveolin-1 expression by siRNA also resulted in marked reduction of β1 integrin endocytosis (Shi and Sottile, 2008). Furthermore, tyrosine-phosphorylated caveolin-1 can promote Rho-activation, FAK activation, and hence focal adhesion disassembly in cancer cells (Joshi et al., 2008; Goetz et al., 2008). Therefore, different

integrins can be trafficked in cells through routes that are both dependent and independent of clathrin.

Transport of integrins through endosomal compartments is also important for cell migration. The Rab family of small GTPases plays important roles in intracellular vesicle trafficking. For example, they ensure the correct cargo is delivered to the right addresses within the cell by recruiting effector proteins (Stenmark, 2009). Based on experiments done in cell culture, several Rab proteins are involved in the current model of integrin trafficking. Integrins were observed to associate with Rab5 after internalization and localize in Rab5 positive early endosomes following focal adhesion disassembly (Pellinen and Ivaska, 2006; Ezratty et al., 2009). Rab5 positive early endosomes act like a sorting station (for review, Grant and Donaldson, 2009). Integrins in early endosomes can be first transferred to the perinuclear compartments, the membrane tubules originated from early endosomes, then transported back to the plasma membrane in recycling endosomes that emanate from perinuclear compartments (Caswell and Norman, 2006; Grant and Donaldson, 2009). This so-called "long-loop" recycling can be regulated by Rab11 and Arf6 (Caswell and Norman, 2006; Powelka et al., 2004; Yoon et al., 2005). Integrins can also be rapidly recycled through a Rab4-dependent "short-loop" recycling without passing through the perinuclear compartment (Roberts et al., 2001).

1.5. Objectives, rationale and hypotheses

Cell culture studies have shown that integrins undergo turnover in migrating cells and that this dynamic property of integrin was essential for the transient, short-term adhesion that underlie integrin-mediated cell migration (Caswell et al., 2009; Pellinen et al., 2008). Although the mechanism of integrin turnover in transient cell adhesion was analyzed in cell culture, how integrin turnover is regulated in living organisms has yet to be studied. Moreover, little attention has been focused on the dynamics and biological significance of integrin turnover at stable, long-term adhesive contacts. In this study, my aim is to elucidate the molecular mechanisms underlying integrin turnover and their biological importance at stable adhesion sites in live organisms. First, I hypothesize that integrins and the IAC are dynamic at *Drosophila* MTJs. Second, I further hypothesize that the mechanism of integrin turnover at MTJs is not due to lateral diffusion but to protein recycling. Moreover, I hypothesize that ectopic perturbation of IAC dynamics will lead to mutant phenotypes at MTJs.

Drosophila muscle tissue undergoes multiple dynamic morphogenetic processes during embryogenesis including cell migration, cell rearrangement and cellular remodeling. Once muscle morphogenesis is complete, muscles undergo dramatic growth, while the MTJs remain in place throughout the remaining embryonic and larval stages, a period lasting over 5 days. In functioning muscles, MTJs withstand tensile force generated by muscle contraction through active integrins that are bound to the ECM and cytoskeleton (Bokel and Brown, 2002). Therefore, I hypothesize that integrin adhesions undergo turnover in MTJs during late embryogenesis and this turnover is down-regulated as

development proceeds from embryos through the larval stages to maintain tissue integrity. Moreover, mechanotransduction was shown to regulate integrin turnover in non-muscle cells; therefore, I hypothesize that tensile force generated by muscle contraction can also regulate integrin turnover at MTJs throughout development. Finally since signaling pathways downstream of integrin and the EGFR exhibit cross talk at multiple levels, I hypothesize that the EGFR pathway plays a role in regulating IAC turnover during development.

CHAPTER 2: MATERIALS AND METHODS

2.1.   Molecular biology

The genomic rescue construct pUbi talin-GFP was made using the pUbi talin vector (Tanentzapf and Brown, 2006). The EGFP coding region was amplified from pEGFP-N1 vector by polymerase chain reaction and MluI restriction enzyme sites were added flanking the CDS. EGFP was then inserted near the end of the talin coding region after amino acid 2756. pUbi βPS integrin-YFP was created from pHSβPS-Venus plasmid (Gift of Tom Bunch) which contains a cDNA clone of βPS integrin with an internal Venus YFP fusion protein inside a non-conserved, serine rich region of the 5' section of the hybrid domain replacing residues 113-134 with a non-native serine and threonine residue followed by the Venus YFP coding sequence. A 4783bp section incorporating the HS70 promoter, the βPS integrin-YFP fusion protein, and the Tubulin PolyA site was excised from the plasmid using the restriction enzyme XbaI and inserted into the SpeI site downstream of the Ubiquitin-63E promoter in the pWRpAUbiP plasmid (Tanentzapf and Brown, 2006).   The HS70 promoter was then excised using SacII and SgrAI. The Y831F/Y843F mutations were then introduced to the plasmid using the QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene). The mutations were introduced through two consecutive rounds of mutagenesis with the following primers (5' only shown, changes in bold): Mutation Y831F, a>t (gcgagaatcccatc/t**t**c/aagcaggccacgtc), and Mutation Y843F, a>t (ccaccttcaagaaccccatg/t**t**t/gcgggcaaat). Transgenics were generated by BestGene Inc. (Chino Hills, CA).

2.2. Fly stocks and lines

Stocks used in this study obtained from the Bloomington Drosophila Stock Centre were: UAS *shi*^{ts}, UAS Rab5.S43N (Rab5^{DN}), UAS Rab5, UAS Rab5.Q88L (Rab5^{CA}), UAS Rab21, UAS Rab21.T27N (Rab21^{DN}), UAS Rab21.Q73L (Rab21^{CA}), UAS Rab2, UAS InR^{CA}, UAS InR^{DN} and Mef2 Gal4. Mef2 Gal4 was used to drive UAS expression in muscles. To visualize core IAC components for FRAP experiments fluorescently tagged integrin and talin (both made by Michael Fairchild in the lab) were expressed under the ubiquitous promoter in flies. Additionally, previously described fly lines with GFP labeled genomic tensin (from Dr. Nick Brown, Cambridge University) and ILK (from the FlyTrap consortium, Yale University) were also used. The temperature sensitive muscle hypercontraction mutants *Breakdance*^{J29} and *Swing*^{w118} were gifts from Dr. Troy Littleton, MIT (Montana and Littleton, 2004). The fly lines used to regulate EGFR signaling pathway were: UAS EGFR^{DN}, *egfr*^{co} (Clifford and Schüpbach, 1989; Kumar et al., 1998), and UAS Yan^{CA} (Gabay et al., 1996). UAS foxo was a gift from Dr. Leann Jones from The Salk Institute for Biological Studies and was described in Flatt et al., 2008. *Para*^{ts2} was a gift from Dr. Troy Littleton and was described by Pittendrigh et al. (Pittendrigh et al., 1997). FAK GFP and FAK Y430F GFP were gifts from Dr. Ruth Palmer, Umea University, Sweden.

2.3.   FRAP experiments and statistical analysis

2.3.1. Confocal microscope settings for FRAP experiments

FRAP was performed on a confocal microscope (FV1000; Olympus) with an UplanSApo 60x/1.35 oil objective (Olympus) and fully opened pinhole. Fluorescence intensity was recorded for 75 frames with an interval of 4 seconds between each two frames. For all experiments other than the flat preparation, photobleaching was performed using the 405-nm laser at 30% power using the Tornado scanning tool (Olympus) for 2 seconds at 100µs/pixel. Obtaining sufficient levels of photobleaching in the whole MTJ FRAP experiments presented in Fig. 2 required the use of the rectangular scanning tool (Olympus) for 2 seconds at 8µs/pixel. Since CPZ treatment resulted in high levels of background noise when exposed to the 405-nm laser, photobleaching experiments were carried out using the 473-nm laser at 30% power for 2 seconds in all experiments involved flat preparations. The laser performance was maximized through software updates and maintenance in March 2010, which results in higher bleaching power under the same settings. To compensate this, bleaching power was adjusted from 30% to 22.5% in an attempt to mimic the bleaching conditions previously used. However, this bleach setting still results in a 5% difference in the averaged mobile fractions of heterozygous integrins. Hence, experiments performed after this point: analyses of force related regulation of integrins (wt and YYFF mutants) in larvae, Yan[CA] experiments, and transheterozygous mtm mutant experiments were compared to controls under the same bleaching conditions.

2.3.2. Whole mount sample preparations

Embryos used for FRAP analysis were collected from apple juice plates and dechorionated in 50% bleach for 5 minutes, washed with PBT followed by PBS. Embryos were then mounted on glass slides in PBS. Larvae used for FRAP analysis were collected from apple juice plates and mounted on glass slides in PBS. FRAP analysis was carried out in room temperature on whole mount embryos or on whole mount larvae 2 hours after mounting.

Experiments using UAS *shi^{ts}*, Rab GTPases constructs, muscle contraction mutants (*Swing^{X118}*, *Breakdance^{J29}* and *Para^{ts2}*), insulin receptor (InR) and epidermal growth factor receptor (EGFR) signaling pathway constructs were carried out in flies heterozygous for βPS integrin-YFP or ILK-GFP to allow combination of multiple alleles or transgenes in one fly. Heterozygous βPS integrin-YFP and ILK-GFP flies exhibit higher levels of turnover compared to homozygous βPS integrin-YFP and ILK-GFP flies of the same stage. All other analyses of βPS integrin-YFP turnover were carried out in homozygous βPS integrin-YFP flies.

2.3.3. Temperature sensitive sample preparations

To study temperature sensitive alleles and transgenes, temperature treatments were performed on both experimental groups (temperature sensitive alleles or transgenes combined with fluorescently tagged integrins or other IAC components) and respective control groups (heterozygous fluorescently tagged integrins or other IAC components).

Because different alleles or transgenes have different sensitivity to temperatures, a variety of temperature sensitive treatments were performed specific to each of the alleles or transgenes. Experimental and control groups used to study the effect of UAS shi^{ts} which were heat shocked (30°C for embryos and 37°C for larvae) for an hour prior to FRAP analysis. FRAP was performed at 30°C or 37°C in an on-stage incubator. Experimental and control groups used to study the effect of $Breakdance^{J29}$, $Swing^{X118}$, and $Para^{ts2}$ were heat shocked at 37°C for two hours prior to FRAP analysis. FRAP was performed at room temperature.

2.3.4. Larval flat preparations

Late third instar larvae were dissected in modified HL3 medium (Kasprowicz et al., 2008) Dissected larval fillets were incubated in Schneider's medium with either 50μM chlorpromazine (CPZ) (Sigma-Aldrich) or 0.4mM dynasore (Sigma-Aldrich) for 30 minutes. As controls, dissected larvae were incubated in Schneider's medium without CPZ or dynasore for 30 minutes. After incubation, fillets were washed with Schneider's medium and mounted (inside facing down) on a glass bottom 35mm petri dish. FRAP was then performed on the mounted fillets at room temperature immediately after mounting.

2.3.5. Statistical analysis

To control for muscle twitching in and out of focus, multiple Regions of Interest (ROI) were selected in non-photobleached regions; only samples in which intensities within

control ROIs remained steady throughout the FRAP experiment were used. Recovery data was further analyzed using Prism (GraphPad, La Jolla CA): mobile fraction and $t_{1/2}$ were calculated as previously described (Reits and Neefjes, 2001), and statistical tests (t-test, ANOVA test) were carried out using Prism.

2.4. <u>Immunohistochemistry and confocal microscopy</u>

Antibody staining was carried out according to standard procedures. Heat fixation (Tanentzapf and Brown, 2006) was used for all embryonic staining to allow staining of late-stage embryos. The following antibodies were used: α-αPS2 (7A10 (Bogaert et al., 1987); rat mAb; 1:10), α-Talin (E16B (Brown et al., 2002), mouse mAb;1:10), α-Tiggrin (Mouse pAb (Fogerty et al., 1994), 1:500; gift of L. Fassler), α-Paxillin (rabbit pAc; 1:500); α-MHC (mouse mAb; 1:200; gift of Dan Kiehart), α-GFP (A6455; rabbit pAb; 1:1000; Invitrogen). Images were collected using Olympus FV1000 confocal microscope with an UplanSApo 60x/1.35 oil objective and processed using Adobe Photoshop.

2.5. <u>Analysis of MTJ width</u>

Live homozygous integrin-YFP embryos and larvae (embryonic stage 17, L2, early L3, late L3) were imaged under identical settings. The width of 14 randomly selected muscle junctions from each of 4 developmental stages was measured.

CHAPTER 3: RESULTS

3.1. <u>Integrin and IAC components undergo turnover at MTJs</u>

To visualize the turnover of integrins in live *Drosophila* embryos and larvae, I used transgenic flies containing fluorescently tagged βPS integrins (βPS integrin-YFP, made by Michael Fairchild, see 2.1). Similarly, three other lines of flies with fluorescently labeled talin, tensin, and ILK were utilized (see 2.1-2.2). All fluorescently tagged transgenes used were able to rescue embryonic muscle phenotypes of null mutations in their respective genes if applicable and faithfully reproduced the expression pattern of the endogenous untagged molecule ((Hudson et al., 2008; Torgler et al., 2004); Tanentzapf Lab data not shown).

FRAP allows the measurement of the mobile fraction, which represents the amount of mobile molecules as a proportion of all fluorescent molecules present (Reits and Neefjes, 2001). I used FRAP to determine the mobile fractions of integrin and core components of the IAC in *Drosophila* MTJs. The MTJ in *Drosophila* embryos and larvae forms a distinct, thin, linear, adhesion site at the ends of the muscle, which is well suited for FRAP analyses (Fig. 6a). FRAP experiments on homozygous βPS integrin-YFP flies revealed that in mature MTJs of late stage embryos the mobile fraction of βPS integrin was 40% (Fig. 6b, Table 1). FRAP experiments on fluorescently labeled IAC components, tensin, talin, and ILK, also revealed the presence of a mobile fraction (Fig.

Figure 6 – **Integrin, talin, ILK and tensin undergo turnover in MTJs:** (a) Time course of recovery of βPS integrin-YFP after photobleaching of one muscle attachment (arrow) in a stage 17 embryo. The other muscle attachments are shown as controls. (b-e) Averaged FRAP recovery curves for βPS integrin-YFP (b), ILK-GFP (c), talin-GFP (d), and tensin-GFP (e) (Each recovery curve is an average of 10 individual FRAP experiments; error bars represent standard error) (Reprinted with permission of the Company of Biologists Ltd: [Journal of Cell Science] Yuan et al., 2010).
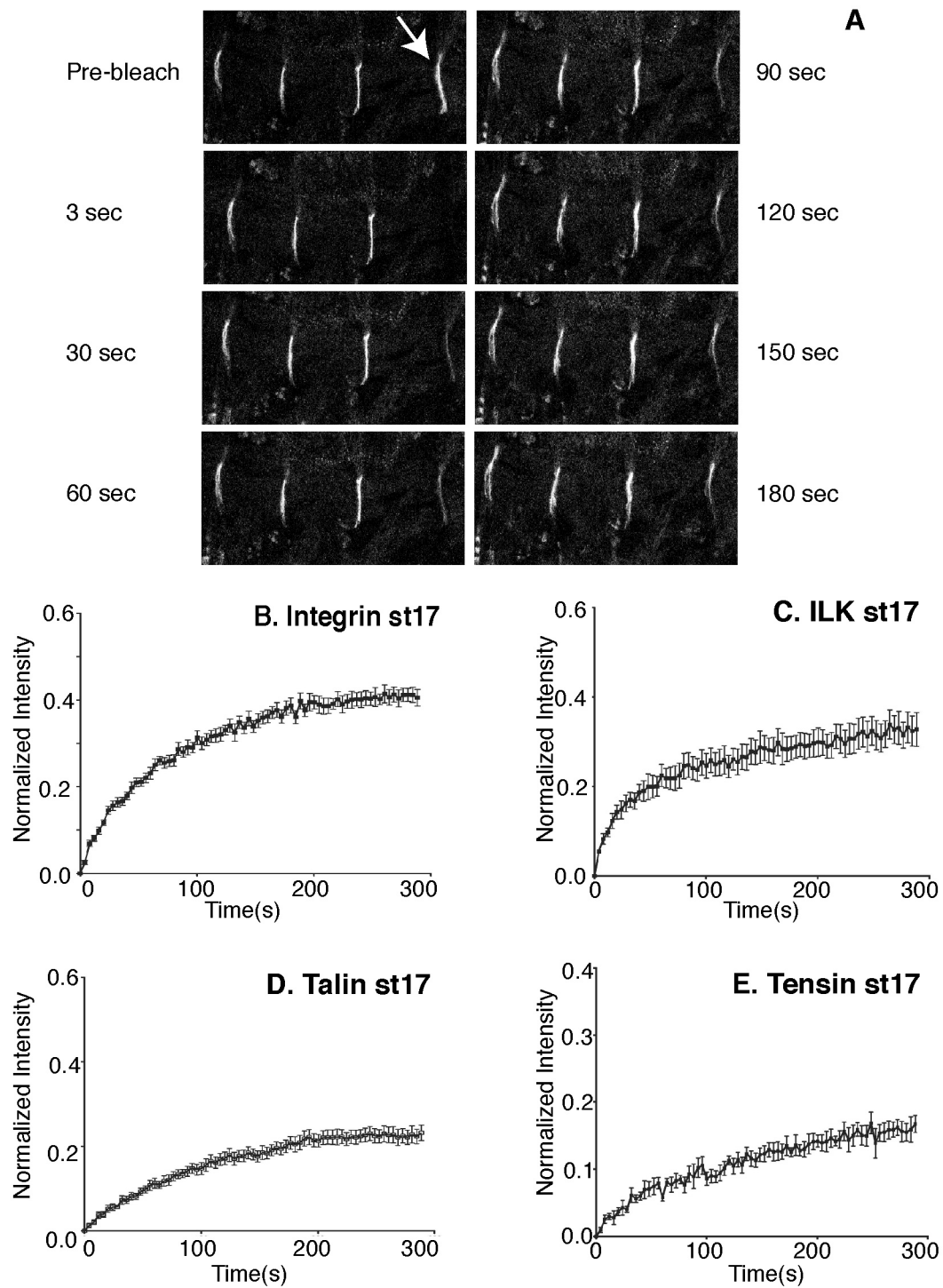
Figure 6 - Integrin, talin, ILK, and tensin undergo turnover in MTJs

Table 1 – **Mobile fractions of integrin, talin, ILK and tensin that undergo turnover in embryonic stage 17 MTJs**

Genotype	MF ES 17 (Mean±SEM)
βPS integrin-YFP/ βPS integrin-YFP	0.401±0.031
+/ILK	0.327±0.038
talin/talin	0.232±0.018
tensin/tensin	0.167±0.013
βPS integrin-YFP/ βPS integrin-YFP[2]	**MF (ES17) (Mean±SEM)**
Small area	0.648±0.045
Entire MTJ	0.653±0.052

Table 1 - Mobile fractions of integrin, talin, ILK and tensin in ES 17 MTJs

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[2] Different bleaching settings were required for the lateral diffusion experiments (see 2.3.1.) and mobile fractions measured under such settings were different than those using standard bleaching settings.

6c-e, Table 1). The $t_{1/2}$ values for all IAC components studied were less than 100 seconds and no recovery occurred beyond 300 seconds after photobleaching; however, $t_{1/2}$ values calculated were not very statistically robust due to the large standard error values (Table 2).

3.2. Integrin and IAC turnover require clathrin-mediated endocytosis

3.2.1. Lateral diffusion

To determine whether the observed mobile fraction was due to lateral diffusion, we compared experiments in which photobleaching was performed on fluorescently labeled βPS integirns (βPS integrin-YFP) at an entire MTJ to experiments in which photobleaching was performed on only a small section of the βPS integrin-YFP at a MTJ (Fig. 7). In both partial MTJ and whole MTJ FRAP experiments, similar mobile fraction values were obtained showing that the fluorescent recovery was unlikely to be due to lateral diffusion (Fig. 7; Table 1). This is consistent with previously published results showing that integrins have low lateral mobility in stable adhesive contacts (Duband et al., 1988). Because lateral diffusion does not contribute to the observed fluorescence recovery of βPS integrin at MTJs, the averaged mobile fraction obtained from βPS integrin FRAP experiments is a measure of βPS integrin turnover. It should be noted that successful photobleaching of an entire muscle attachment requires adjustment of the FRAP settings (see 2.3.1.). These adjustments lead to the observation of different values for the mobile fraction than other FRAP experiments using homozygous βPS integrin-YFP flies (compare to Fig. 7b; Table 1).

Table 2 – $t_{1/2}$ **values of integrin and ILK turnover in flies expressing** *shi*[ts]**, Rab5**[DN]**, Rab5**[CA]**, or treated with dynasore, or CPZ** (Reprinted with permission of the Company of Biologists Ltd: [Journal of Cell Science] Yuan et al., 2010)

Genotype	$t_{1/2}$(L3) (sec)(Mean±SEM)
ILK-GFP /+	74.75±44.84
UAS-Rab5[DN] /ILK-GFP; Mef Gal4	71.00±38.49
UAS-Rab5[CA] /ILK-GFP; Mef Gal4	38.24±18.54
UAS-Rab5 /ILK-GFP; Mef Gal4	40.85±18.36
UAS-Rab2 /ILK-GFP; Mef Gal4	35.64±23.49
UAS-Rab21[CA] /ILK-GFP; Mef Gal4	44.85±32.65
UAS-Rab21[DN] /ILK-GFP; Mef Gal4	45.73±16.78
ILK-GFP/+	66.59±57.65
UAS-*shi*[ts] /ILK-GFP; Mef Gal4	34.37±25.82
βPS-integrin-YFP/+	66.47±18.47
UAS-Rab5[DN]/ βPS-integrin-YFP; Mef Gal4	58.58±15.78
UAS-Rab5[CA] / βPS-integrin-YFP; Mef Gal4	59.45±18.36
βPS-integrin-YFP /+	53.49±16.33
UAS-*shi*[ts] / βPS-integrin -YFP; Mef Gal4	52.71±12.05

Table 2 - t1/2 values from the FRAP analysis of integrin and ILK turnover in flies expressing *shi*[ts]**, Rab5**[DN]**, Rab5**[CA]**, or treated with dynasore or CPZ**

(Table 2 continues)

Larval Flat Preparation	$t_{1/2}$(L3)(sec) (Mean±SEM)
βPS-integrin-YFP (control)	18.99±17.38
βPS-integrin-YFP (CPZ)	47.99±13.05
βPS-integrin-YFP (dynasore)	53.92±44.21
ILK-GFP (control)	8.517±4.803
ILK-GFP (CPZ)	30.71±31.56
ILK-GFP (dynasore)	22.07±22.90

Table 2 - t1/2 values from the FRAP analysis of integrin and ILK turnover in flies expressing *shi*[ts],

Rab5[DN], Rab5[CA], or treated with dynasore or CPZ

Figure 7 –**Integrins do not exhibit lateral mobility**: (a, b) Averaged FRAP recovery curves in intact live 3rd instar larva for integrin-GFP are nearly identical (n=12, p=0.9462, two-tailed t test) when the entire MTJ is photobleached or if only a small section of the junction is photobleached. (c) As *Drosophila* embryonic MTJs are typically thin in depth, photobleaching occurs efficiently in each plane of the MTJ. Total Z-stack thickness is 3μm, with each focal plane 0.6μm apart (Reprinted with permission of the Company of Biologists Ltd: [Journal of Cell Science] Yuan et al., 2010).
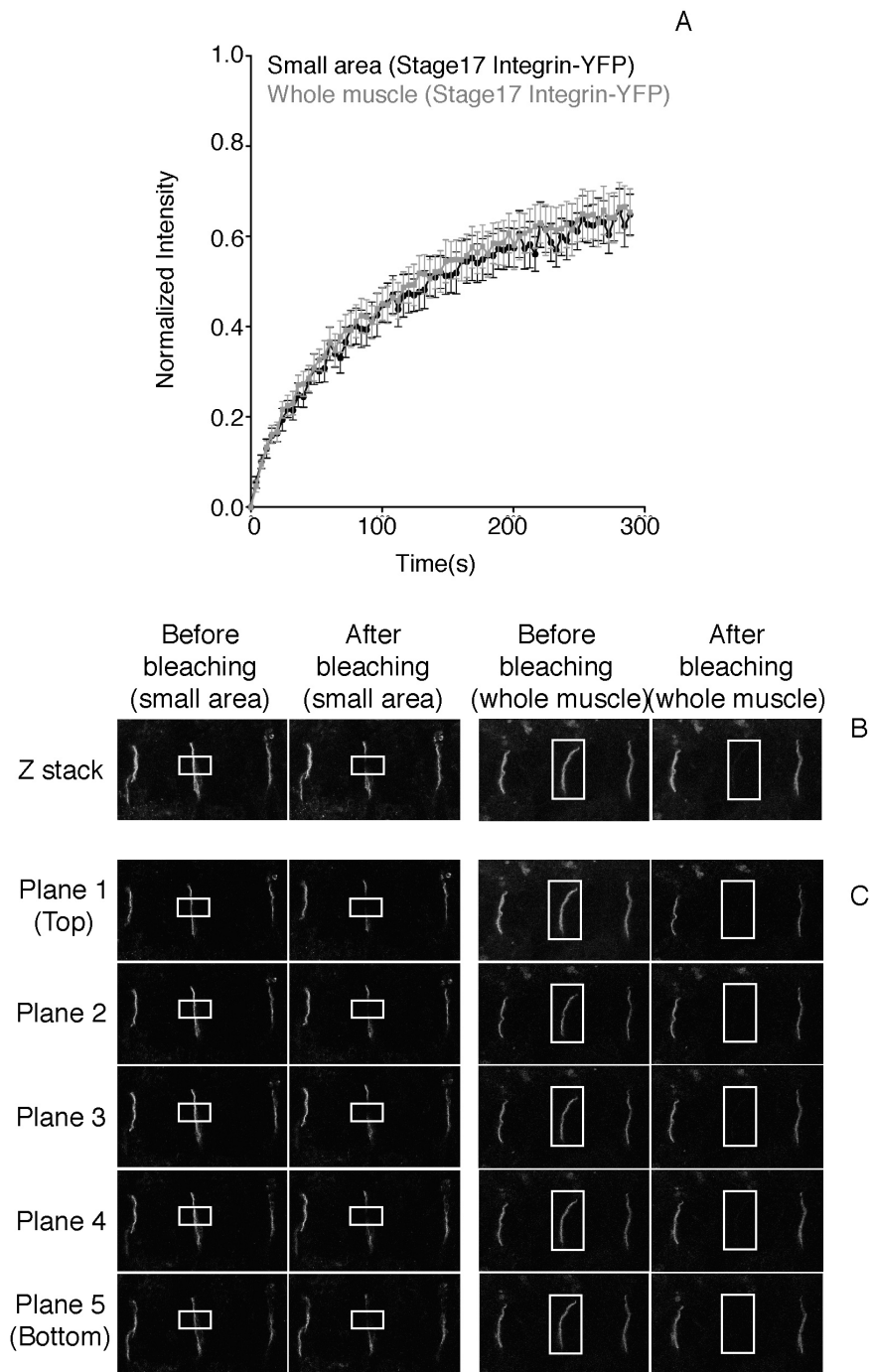
Figure 7 - Integrins do not exhibit lateral mobility

3.2.2. Endocytosis

To explore the mechanisms underlying integrin turnover in MTJs a mutant temperature sensitive allele of dynamin (UAS-*shi*[ts]) was used to inhibit endocytosis in fly muscles (Kitamoto, 2001). I expressed UAS-*shi*[ts] in muscle specific tissues and used high temperature to reduce dynamin activity in muscles (see Materials and Methods). I found that our control heterozygous βPS integrin-YFP flies exhibited a slightly higher recovery when kept at the high temperature but that otherwise the FRAP curves were unaffected. Higher recovery may be resulted from the duration of exposure to high temperature, which was one hour compared to the standard two-hour incubation time in room temperature (see 2.3.3.). When dynamin function was reduced using muscle-specific expression of *shi*[ts] this led to a small but statistically significant reduction of the averaged mobile fractions of both βPS integrin and ILK (Fig. 8a, b; Table 3). The small effect conferred by UAS-*shi*[ts] is likely the result of the limitations of using an ectopic, temperature sensitive construct in an otherwise wildtype background.

To confirm the role of endocytosis in integrin turnover, I took a pharmacological approach using the dynamin inhibitor dynasore (Macia et al., 2006). Open-book dissections were performed on late 3rd instar larvae, which allow us to treat muscles and epidermis containing intact MTJs with pharmacological agents and to study changes in protein turnover using FRAP (see 2.3.4.). These flat preparations exhibited normal muscle contractions for a few hours following dissection and mounting and the

Figure 8 – **Integrin turnover at MTJs requires clathrin-mediated endocytosis:** (a,b) Averaged FRAP recovery curves in intact live 3rd instar larva for βPS integrin-YFP/+ (a) and ILK-GFP/+ (b) in a wildtype background and in muscles expressing a UAS-*shi*[ts] transgene. (c-f) Averaged FRAP recovery curves for βPS integrin-YFP (c,e) and ILK-GFP (d,f) in a 3rd instar wildtype larval flat prep with or without dynasore (c,d) or chlorpromazine (CPZ) (e,f). (Statistical analysis of significance of differences between averaged FRAP curves by Two-tailed t-test: (a) n=14, p = 0.0012; (b) n=10, p=0.0303; (c) n=7, p=0.0004; (d) n=7, p=0.0012; (e) n=7, $p<0.0001$; (f) n=8, p=0.0009; error bars represent standard error) (Reprinted with permission of the Company of Biologists Ltd: [Journal of Cell Science] Yuan et al., 2010).

A. Integrin; *shibere*^ts

B. ILK; *shibere*^ts

C. Integrin treated with Dynasore

D. ILK treated with Dynasore

E. Integrin treated with CPZ

F. ILK treated with CPZ

Figure 8 - Integrin turnover at MTJs requires clathrin-mediated endocytosis

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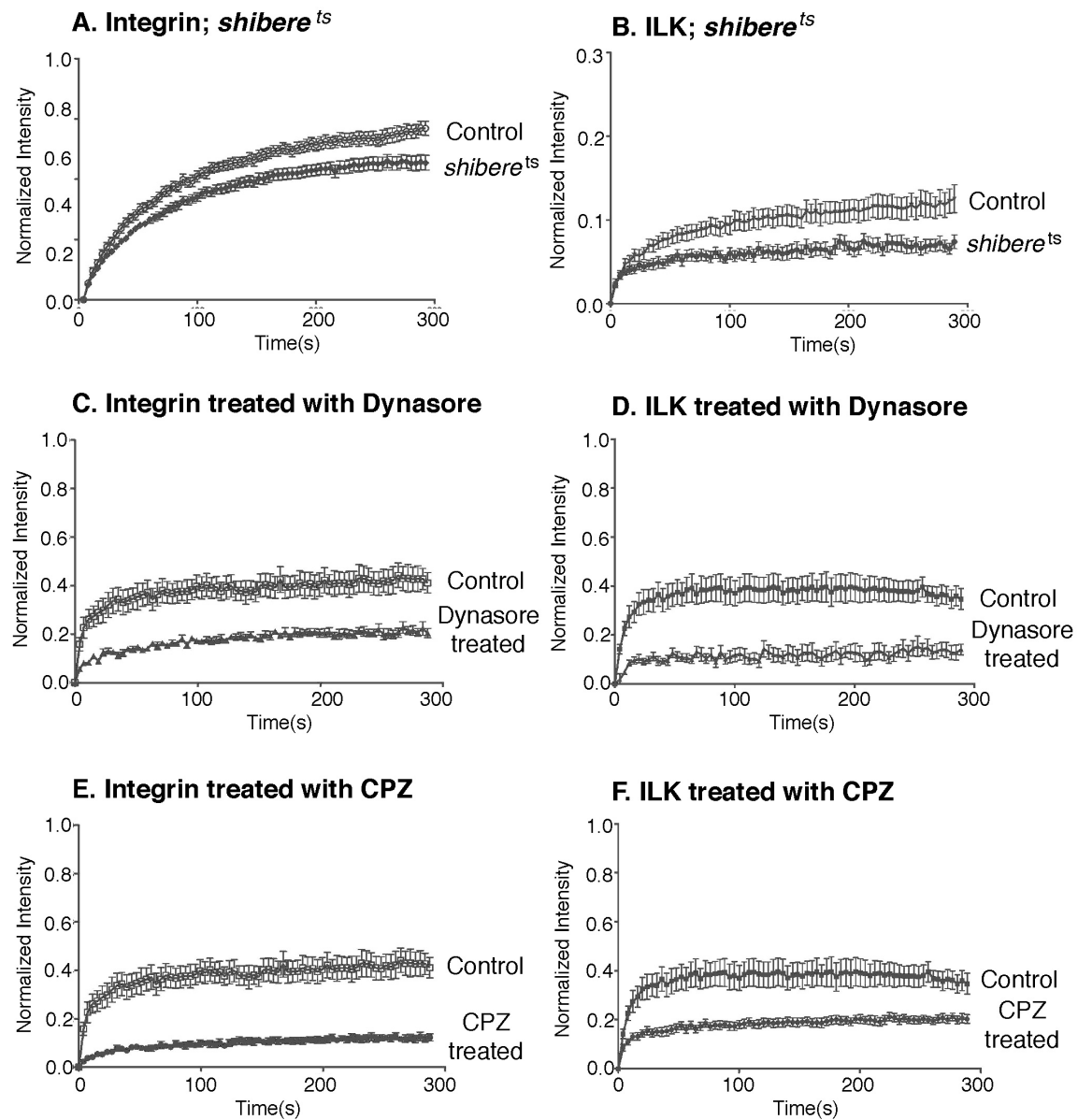
Table 3– **Mobile fractions of βPS-Integrin-YFP, and ILK-GFP flies expressing *shi*[ts],
Rab5[DN], Rab5[CA], or treated with dynasore, or CPZ** (Reprinted with permission of the
Company of Biologists Ltd: [Journal of Cell Science] Yuan et al., 2010).

Intact Early L3 Genotype	MF (L3) (Mean±SEM)
ILK-GFP /+	0.130±0.029
UAS-Rab5[DN] /ILK-GFP; Mef Gal4	0.047±0.013
UAS-Rab5[CA] /ILK-GFP; Mef Gal4	0.211±0.012
UAS-Rab5 /ILK-GFP; Mef Gal4	0.125±0.017
UAS-Rab2 /ILK-GFP; Mef Gal4	0.159±0.020
UAS-Rab21[CA] /ILK-GFP; Mef Gal4	0.127±0.025
UAS-Rab21[DN] /ILK-GFP; Mef Gal4	0.132±0.018
ILK-GFP/+	0.134±0.024
UAS-*shi*[ts] /ILK-GFP; Mef Gal4	0.074±0.008
βPS integrin-YFP/+	0.607±0.038
UAS-Rab5[DN]/ βPS integrin-YFP; Mef Gal4	0.534±0.027
UAS-Rab5[CA] / βPS integrin-YFP; Mef Gal4	0.760±0.021
βPS integrin-YFP /+	0.697±0.026
UAS-*shi*[ts] / βPS integrin -YFP; Mef Gal4	0.547±0.036

**Table 3 - Mobile fractions of integrin-YFP and ILK-GFP flies expressing *shi*[ts], Rab5[DN], Rab[CA], or
treated with dynasore or CPZ**

(Table 3 continues)

Larval Flat Preparation	MF (L3) (Mean±SEM)
βPS integrin-YFP (control)	0.411±0.042
βPS integrin-YFP (CPZ)	0.123±0.014
βPS integrin-YFP (dynasore)	0.204±0.019
ILK-GFP (control)	0.377±0.047
ILK-GFP (CPZ)	0.203±0.018
ILK-GFP (dynasore)	0.141±0.022

Table 3 - Mobile fractions of integrin-YFP and ILK-GFP flies expressing shi, Rab5[DN], Rab[CA], or

treated with dynasore or CPZ

intensity of both ILK-GFP and βPS integrin-YFP recovered following photobleaching. I noticed that mobile fractions observed in control (untreated) MTJs were lower for βPS integrin and higher for ILK when compared to that seen in intact larvae of the same genotype (Fig. 8c,d). This is likely due to the fact that these experiments required the use of different FRAP settings (see 2.3.1.) but might reflect innate differences between flat prep culture and *in vivo* conditions. When 3rd instar larval flat preparations were treated with dynasore, a small cell permeable molecule that specifically inhibit dynamin functions (Macia et al., 2006), the averaged mobile fractions of ILK and βPS integrin were reduced by 63% and 50% respectively (Fig. 8c,d).

Because dynamin can mediate both clathrin-dependent and clathrin-independent endocytosis (Caswell and Norman, 2006; Pellinen and Ivaska, 2006), we used chlorpromazine (CPZ), an inhibitor specific to clathrin-dependent endocytosis in both cell culture systems and in *Drosophila* flat prep larvae, to study if clathrin-mediated endocytosis contributes to integrin and IAC turnover (Balzac et al., 2005; Blitzer and Nusse, 2006; Trushina et al., 2006; Wang et al., 1993). In CPZ treated 3rd instar larval flat preparations the mobile fractions of ILK and βPS integrin declined by 46% and 70% respectively (Fig. 8e,f). These results indicate that integrin turnover in stable adhesions requires active clathrin-dependent endocytosis of integrins from the plasma membrane.

3.2.3. Rab5 regulates IAC turnover

The Rab family of small GTPases is central in intracellular vesicle trafficking. They ensure correct cargos are delivered to the right addresses within the cell by recruiting effector proteins (for review, Stenmark, 2009). In experiments done in cell culture, several Rab proteins were showed to involve in integrin trafficking (see 1.4.3). Especially, Rab21 and Rab5 can mediate integrin trafficking following endocytosis at focal adhesions (Pellinen and Ivaska, 2006; Ezratty et al., 2009).

I tested the effects of dominant negative and constitutively active forms of the Rab21 (Rab21$^{DN/CA}$) and Rab5 (Rab5$^{DN/CA}$) in fly muscles (Zhang et al., 2007). Muscle specific expression of neither Rab21^{DN} nor Rab21^{CA} altered ILK dynamics at MTJs (Fig. 9b; Table 3). However, a 12% decrease in the mobile fraction of βPS integrin and a 36% decrease in the mobile fraction of ILK were measured in Rab5^{DN} expressing muscles (Fig. 9c, d; Table 3), which is consistent with the role of Rab5 in IAC turnover described previously (Pellinen et al., 2006; Ezratty et al., 2009). A population of Rab5 positive vesicles was observed to concentrate near the MTJ and overlap with integrins in embryonic muscles (Fig. 9e, f). Moreover, the activation of Rab5 has been shown to increase the motility of endosomes along microtubules in migrating cells (Dinneen and Ceresa, 2004; Nielsen et al., 1999). I tested the effects of expressing a constitutively active Rab5 (Rab5^{CA}) in fly muscles and found that it conferred an increase in the mobile fraction of βPS-integrin and ILK, by 25% and 62% respectively, suggesting that Rab5 activation also promotes IAC turnover at stable adhesions (Fig. 9c, d; Table 3). In

Figure 9 – **Characterization of the effects of Rab5^{CA} and Rab5^{DN} on IAC turnover:**
(a-d) Averaged FRAP recovery curves in intact live 3rd instar larva for ILK-GFP/+ (a-c)
and βPS integrin-YFP/+ (d) in a wildtype background and in muscles expressing Rab
transgenes. (a) Expression of Rab2 (as a control) or Rab5 did not affect the recovery of
ILK-GFP. (b) Expression of constitutively active (CA) or dominant negative (DN) Rab21
does not change the recovery of ILK-GFP following photobleaching. (c, d) Expression of
constitutively active (CA) or dominant negative (DN) Rab5 affects the recovery of ILK-
GFP (c) and integrin-YFP (d) following photobleaching by increasing (Rab5^{CA}) and
decreasing (Rab5^{DN}) the mobile fraction. (e) Rab5 (green, white in e'') is concentrated at
MTJs (arrowheads) where it colocalizes with αPS2 integrin (red, white in e'). (f) Rab5^{CA}
(green, white in f'') increases the width of integrin distribution (αPS2 in red, white in f')
at the MTJs (arrowheads). Statistical analysis of significance of differences between
averaged FRAP curves by one-way ANOVA test: (a) n=10, p=0.4721, (b) n=13,
p=0.4054 (c) n=10, p<0.0001 and (d) n=18, p<0.0001. One-tailed t-test (c) ILK vs
Rab5^{CA} ILK: p=0.0054; ILK vs Rab5^{DN} ILK: p=0.0082; (d) Integrin vs Rab5^{CA} Integrin:
p= 0.0008; Integrin vs Rab5^{DN} Integrin: p= 0.07; error bars represent standard error
(Reprinted with permission of the Company of Biologists Ltd: [Journal of Cell Science]
Yuan et al., 2010).

Figure 9 – Characterization of the effects of Rab5^{CA} and Rab5^{DN} on IAC turnover

comparison, expression of a wildtype form of Rab5 or the secretory pathway regulator Rab2 did not change the mobile fraction of ILK (Fig. 9a; Table 3).

3.3. <u>Integrin turnover and muscle maintenance</u>

Inhibiting integrin turnover in migrating cells stalls cell migration (Woods et al., 2004; White et al., 2007). I therefore analyzed the effect of altering integrin turnover in *Drosophila* MTJs using $Rab5^{CA}$ and $Rab5^{DN}$. I found that expressing either $Rab5^{CA}$ (Fig. 10b, e, h) or $Rab5^{DN}$ (Fig. 10c, f, i) induced muscle-ECM attachment defects in some muscles. Using whole embryo confocal image stacks to identify detached muscles, I found that 27% of embryos expressing $Rab5^{CA}$ (n=41) and 54% of embryos expressing $Rab5^{DN}$ (n=37) have more than one detached muscle. These defects were not due to a failure in IAC assembly but rather to a separation between the integrins at the end of the muscles and the ECM. This is illustrated by the observation that in muscles overexpressing $Rab5^{CA}$ or $Rab5^{DN}$ the IAC markers talin and paxillin are recruited to muscle ends normally but a larger than normal gap can be observed separating integrin and its ECM marker Tiggrin (Fig. 10b,c,e,f). Such defects are consistent with weakening of the connection between integrins and the ECM (Tanentzapf and Brown, 2006). This phenotype arises in late embryonic or early larval stages and is subtler than the dramatic muscle detachment phenotypes caused by the complete loss of integrin-mediated adhesion in embryos (Brown et al., 2000; Tanentzapf and Brown, 2006). Moreover, wider and more diffuse integrin expression was observed in muscles expressing $Rab5^{CA}$ (Fig. 9f). These results show that Rab5 regulates the strength and shape of the MTJs and

Figure 10 – **Expression of Rab5^{CA} and Rab5^{DN} in muscles induces detachment of integrins from the ECM:** (a) In wildtype muscle aPS2 integrin (green) overlaps with the ECM (marked with Tiggrin, red). (b,c) Expression of Rab5^{CA} (b) or Rab5^{DN} (c) led to separation between integrin and the ECM (arrowheads). (d) The ends of the muscle, marked with talin (green), are in contact with the ECM (marked with Tiggrin, red) in wildtype muscles. (e,f) Expression of Rab5^{CA} (e) or Rab5^{DN} (f) led to separation between muscle ends and the ECM (arrowheads). (g) The IAC protein Paxillin (red) colocalizes with aPS2 integrin (green) at the MTJ; muscle is highlighted with MHC (blue). (h,i) Expression of Rab5^{CA} (e) or Rab5^{DN} (f) led to muscle detachment (arrowheads) but did not affect the localization of Paxillin to the muscle end (all muscles are stage 17 embryos) (Reprinted with permission of the Company of Biologists Ltd:[Journal of Cell Science] Yuan et al., 2010).

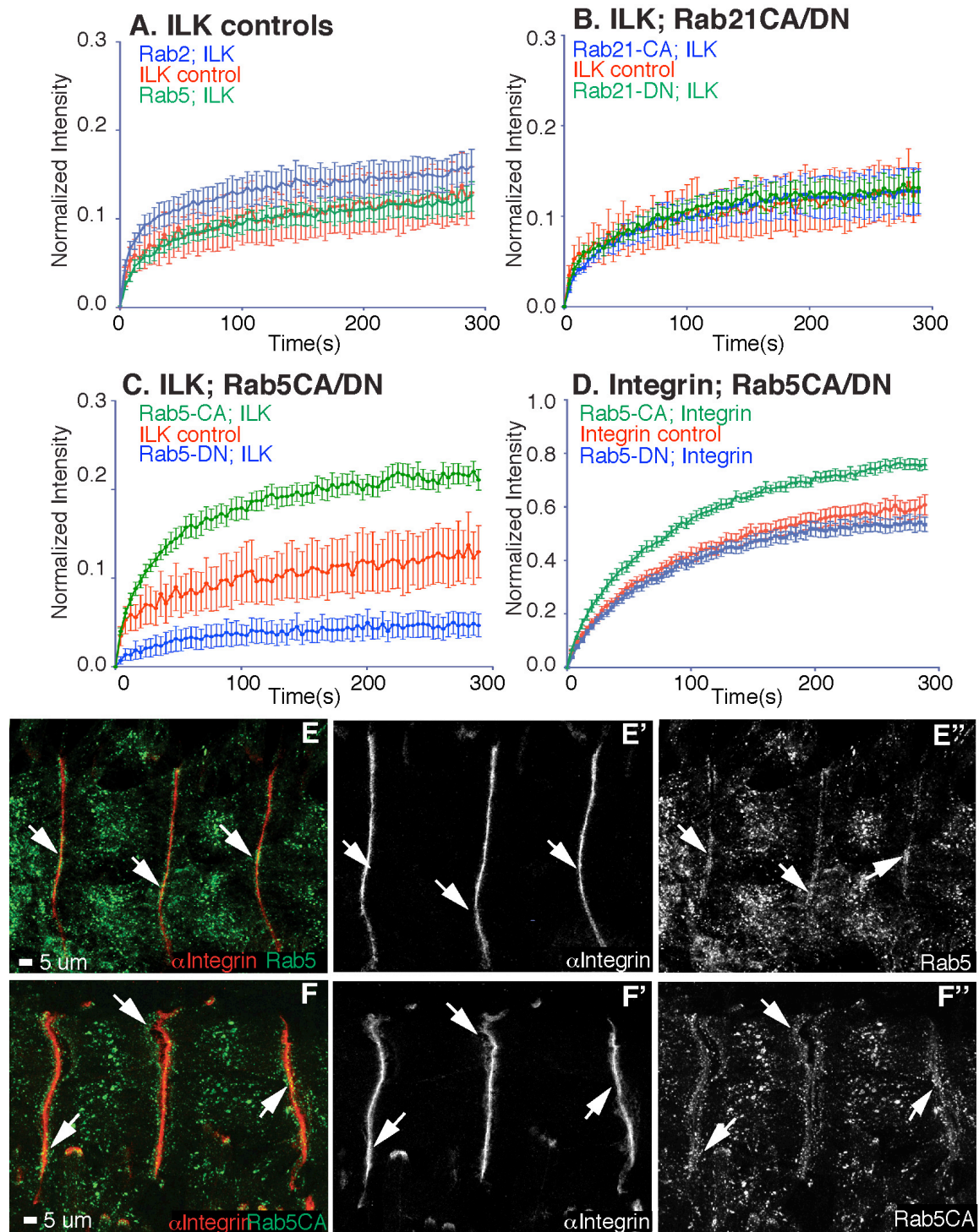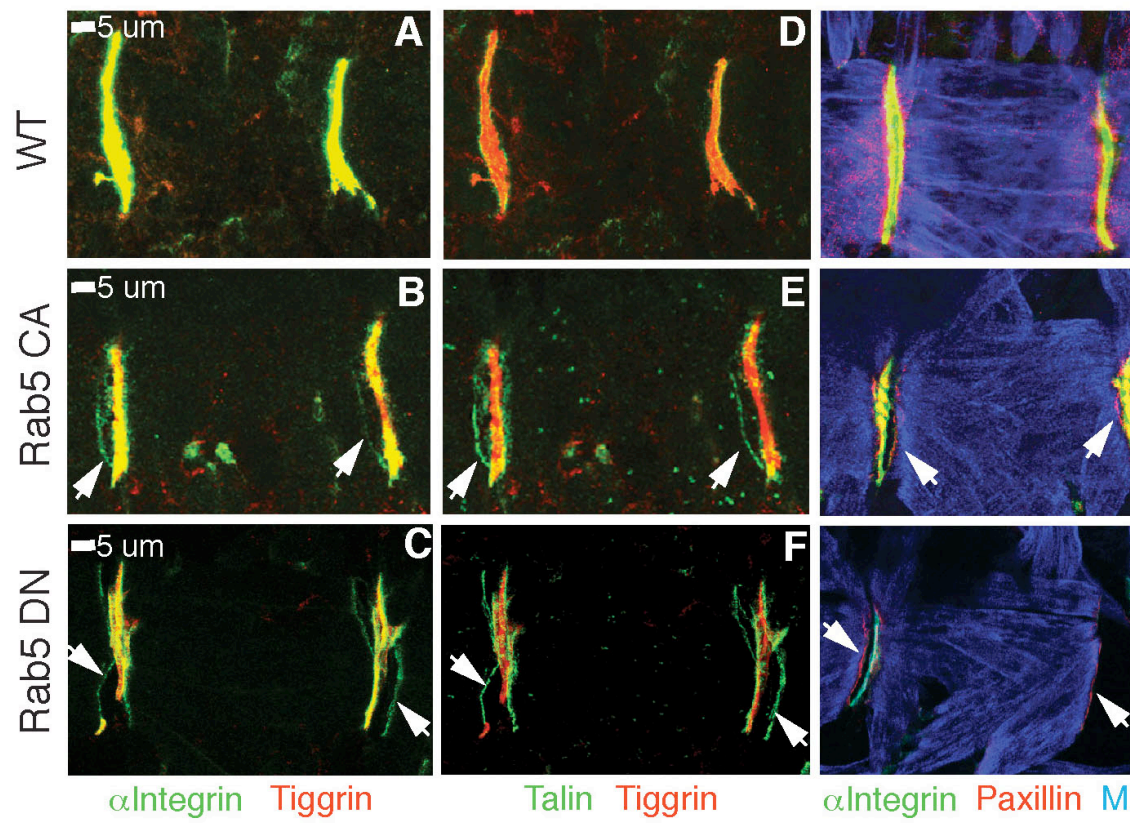**Figure 10 - Expression of Rab5^{CA} and Rab5^{DN} in muscles induces detachment of integrins from the ECM**

that regulation of integrin turnover is essential for maintaining MTJ integrity. In addition, because MTJ attachment defects could potentially affect the mobile fraction, all the FRAP experiments with Rab5^{CA} and Rab5^{DN} were carried out in normally attached muscles.

3.4. IAC turnover at MTJs is developmentally regulated

The *Drosophila melanogaster* MTJ is a well-established model to study integrin-mediated cell-ECM adhesions in the context of development and morphogenesis. Drosophila muscle tissue undergoes multiple dynamic morphogenetic processes during embryogenesis including cell migration, cell rearrangement and cellular shape changes. Once muscle morphogenesis is complete (stage 16), muscles undergo dramatic growth while the MTJs remain in place through the remaining embryonic and larval stages, a period spanning over 5 days. By the end of the 5-day-period, muscle volume has increased 15 times accompanied with an increase in MTJ width by a factor of 4 (Fig. 11a). Moreover, MTJs use integrin-mediated attachments in order to withstand large tensile force generated by muscle contractions (Bokel and Brown, 2002). As growth of MTJs takes place in active and contracting muscles, a mechanism must be in place to ensure simultaneous MTJ stability and growth. The processes that underlie MTJ growth in the larva are not well understood but I hypothesized that they involve a change in the dynamics of integrin turnover. More specifically, I hypothesize that IAC components undergo rapid turnover in MTJs during late embryogenesis, and this IAC turnover is subsequently down-regulated in the embryonic and larval stages to maintain tissue integrity.

Figure 11 – **Stage-specific reduction in IAC mobility at MTJs:** (a, a') Quantification of MTJ width at progressive stages of larval stage development, MTJ width increases by a factor of 4 on average during this time. Average FRAP recovery curves (b-e) and corresponding mobile fraction values (b'-e') for βPS integrin-YFP, ILK-GFP, talin-GFP, and tensin-GFP at progressive developmental stages. Recovery following photobleaching decreases over the course of development. Differences between stages are statistically significant by one-way ANOVA test with the following P-values: (a') n=14, p<0.0001; (b') n =10, p<0.0001; (c') n =10, p<0.0001; (d') n =7, p<0.0001; (e') n =10, p<0.0001; scale bar in (a) is 5 um, and all error bars represent standard error (Reprinted with permission of the Company of Biologists Ltd: [Journal of Cell Science] Yuan et al., 2010).
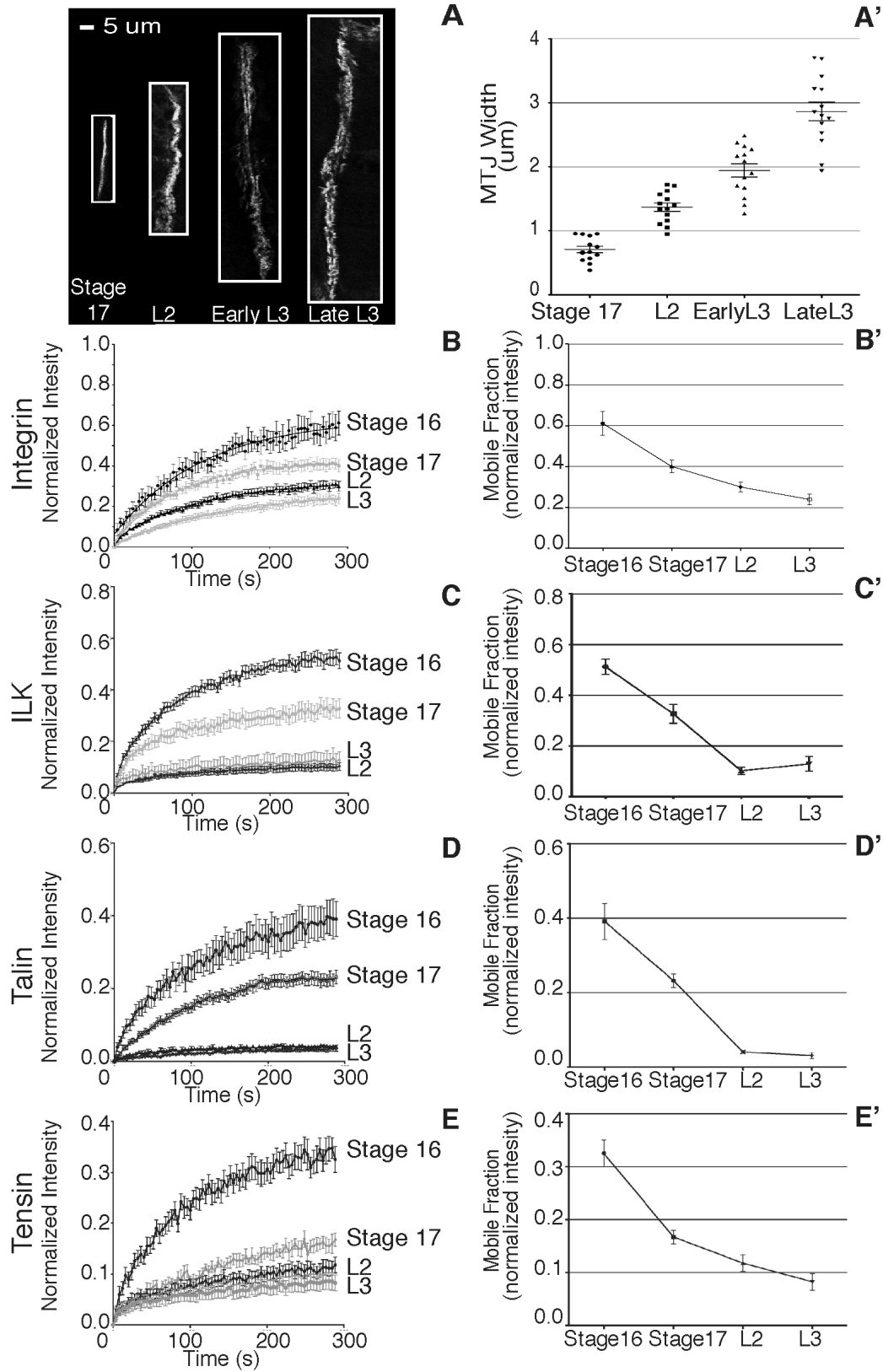
Figure 11 - Stage-specific reduction in IAC mobility at MTJs

Growth-dependent decreases in the mobile fractions of integrin and IAC components were found following MTJ formation (Fig. 11b-e; Table 4). This decline begins during embryogenesis as the mobile fractions of βPS integrin, ILK, talin and tensin decreased by 35%, 36%, 45%, and 49% respectively between stage 16 of embryogenesis, shortly after muscles form, and stage 17, the final stage of embryogenesis. This trend continues so that the mobile fractions of βPS integrin, ILK, talin and tensin decreased by 61%, 76%, 80% and 75% respectively between stage 16 of embryogenesis and 3rd instar larva (Table 4). Nonetheless, even in 3rd instar larva a persistent low level of IAC turnover at the MTJ remains, this level of turnover can be as high as 24% in the case of homozygous βPS integrin-YFP (Table 4).

3.5.   Analysis of candidate growth dependent regulators of integrin turnover

3.5.1. Overview

I have shown using FRAP, that integrin and other core structural components of the IAC (Talin, Tensin and ILK) are dynamic and undergo turnover in MTJs during the time period extending between MTJ formation in late embryogenesis and puparium formation (Fig. 11). Moreover, stage-dependent decreases in the mobile fraction of fluorescently labelled integrin and IAC components were also observed at each subsequent developmental stage. This temporal regulation of integrin turnover may be in place to

Table 4 – **Mobile fractions of integrin, talin, ILK and tensin at different developmental stages**

Genotype (Developmental Stages)	MF (Mean±SEM)
βPS integrin-YFP/ βPS integrin-YFP (ES 16)	0.611±0.058
βPS integrin-YFP/ βPS integrin-YFP (ES 17)	0.401±0.031
βPS integrin-YFP/ βPS integrin-YFP (L2)	0.300±0.024
βPS integrin-YFP/ βPS integrin-YFP (early L3)	0.239±0.025
ILK-GFP/ILK-GFP (ES 16)	0.228±0.006
ILK-GFP/ILK-GFP (ES 17)	0.084±0.056
ILK-GFP/ILK-GFP (L2)	0.041±0.003
ILK-GFP/ILK-GFP (early L3)	0.056±0.008
+/ILK-GFP (ES 16)	0.513±0.031
+/ILK-GFP (ES 17)	0.327±0.038
+/ILK-GFP (L2)	0.102±0.015
+/ILK-GFP (early L3)	0.102±0.011
talin-GFP /talin-GFP (ES 16)	0.391±0.048
talin-GFP /talin-GFP (ES 17)	0.232±0.018
talin-GFP /talin-GFP (L2)	0.040±0.004
talin-GFP /talin-GFP (early L3)	0.036±0.008
tensin-GFP /tensin-GFP (ES 16)	0.325±0.025
tensin-GFP /tensin-GFP (ES 17)	0.167±0.013
tensin-GFP /tensin-GFP (L2)	0.118±0.015
tensin-GFP /tensin-GFP (early L3)	0.082±0.016

Table 4 - Mobile fractions of integrin, talin, ILK and tensin at different developmental stages

maintain tissue integrity, because muscle phenotypes were observed when the IAC turnover was ectopically up or down-regulated (Fig. 11).

To study mechanisms underlying the growth dependent decrease of integrin and IAC turnover, I took a candidate approach. It is possible that signaling pathways known to regulate development, such as the Epidermal Growth Factor Receptor (EGFR) pathway or the Insulin Receptor (InR) pathway, regulate muscle growth and growth dependent decrease of integrin and IAC turnover (Yarnitzky et al., 1998; Demontis and Perrimon, 2009). Alternatively, stage-dependent changes in integrin and IAC turnover could be a secondary consequence of muscle growth. For example, the increasing tension imposed on MTJs by increasingly large and complex muscles could contribute to integrin turnover.

I studied whether either or both of these two factors, signaling pathways that regulate muscle growth and mechanical tension, regulates IAC turnover at MTJs. This was done by analyzing the averaged mobile fractions of integrin and other IAC components in flies with altered EGFR signaling. Also, I used temperature sensitive mutants that can induce muscle hypercontraction or muscle relaxation in combination with FRAP to test the role of mechanical tension in regulating IAC turnover (Montana and Littleton, 2004; Pittendrigh et al., 1997).

3.5.2. The Epidermal Growth Factor Receptor (EGFR) pathway

To test whether the EGFR signaling pathway modulates the developmental down-regulation of IAC turnover, I utilized three different methods to inhibit the EGFR signaling pathway. First, I genetically combined *egfr^{co}*, a null allele of the fly homologue of EGFR, with βPS integrin-YFP (Clifford and Schüpbach, 1989; Kumar et al., 1998). FRAP data showed that integrin dynamics was not affected by the decreasing EGF signaling (p=0.5254, two-tailed t-test with Welch corrections) (Fig. 12a,b; Table 5). I confirmed this result using ectopic expression of dominant negative version of EGFR (UAS EGFR^{DN}). Similarly, no statistically significant change in integrin turnover was detected by FRAP (p=0.2571, two-tailed *t*-test with Welch corrections) (Fig. 12a,b; Table 5).

To further check the possibility that the EGF signaling pathway regulates integrin turnover, I over-expressed a constitutively active version of Yan, a transcriptional repressor that acts downstream of the EGFR pathway, in muscles using the UAS/GAL4 system. Embryonic lethality was observed when UAS Yan^{CA} was expressed in muscles. Despite a severe embryonic phenotype and subsequent embryonic lethality, the muscle specific expression of UAS Yan^{CA} does not perturb the dynamics of integrin in embryonic MTJs (p=0.4652, two-tailed *t*-test with Welch corrections) (Fig. 12c,d; Table 5). In summary, FRAP analysis showed that the developmental regulation of integrin turnover occurs via a process that does not involve the EGF pathway.

Figure 12 – **Characterization of integrin turnover when EGFR signaling is altered:** Averaged FRAP recovery curves (a,c) and averaged mobile fractions (b,d) of βPS integrin-YFP/+ in larval muscles overexpressing *egfr^{co}* mutant allele (a,b) or EGFR^{DN} transgene (a,b) or embryonic muscles overexpressing Yan^{CA} transgene (c,d) measured no differences of βPS integrin turnover compared to βPS integrin turnover in wildtype larval or embryonic muscles. Statistical analysis of significance of differences between averaged FRAP curves by two-tailed t-test: (a) +/βPS integrin vs *Egfr^{co}*/βPS integrin: n=19, 12; p=0.5254 with Welch correction; +/βPS integrin vs EGFR^{DN}/βPS integrin: n=19, 7; p=0.2571 with Welch correction; (b) +/βPS integrin vs Yan^{CA}/βPS integrin: n=16, 20; p=0.4652 with Welch correction. Error bars represent standard error.
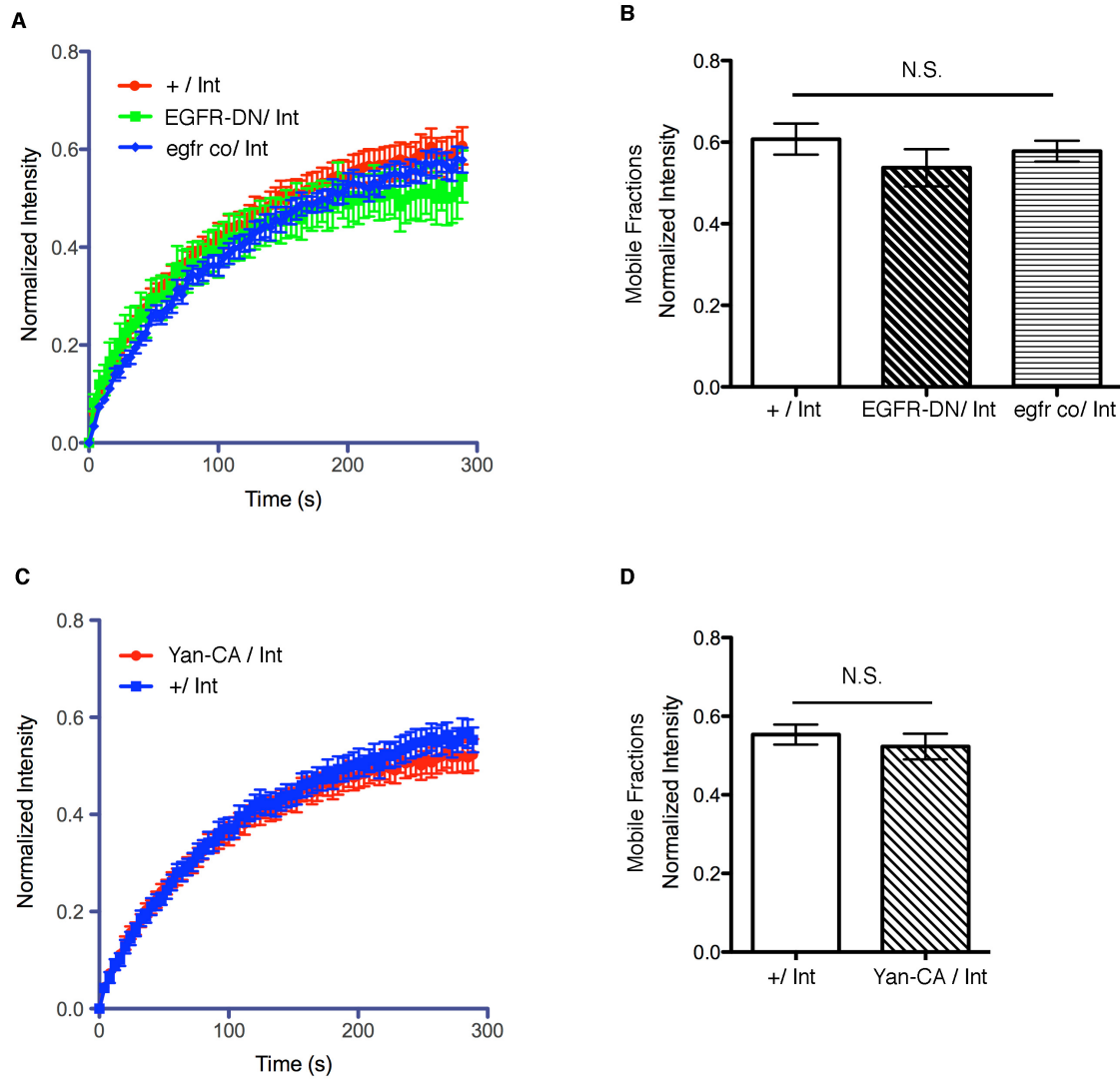
Figure 12 – Characterization of integrin turnover when EGFR signaling is altered

Table 5 - **Mobile fractions of integrin turnover in flies expressing EGFR^{DN}, *egfr^{co}*, and Yan^{CA}**

Genotype (Developmental Stages)	MF (Mean±SEM)
+/ βPS integrin-YFP (early L3)	0.607±0.038
UAS EGFR^{DN} / βPS integrin-YFP; Mef Gal4 (early L3)	0.537±0.045
egfr^{co}/ βPS integrin-YFP (early L3)	0.578±0.025
+/ βPS integrin-YFP; Mef Gal4 (ES 17)	0.554±0.025
UAS Yan^{CA}/ βPS integrin-YFP; Mef Gal4 (ES 17)	0.523±0.033

Table 5 - Mobile fractions of integrin turnover in flies expressing EGFR^{DN}, egfr^{co}, and Yan^{CA}

3.5.3. Tensile force generated by muscle contractions

3.5.3.1.   *Overview*

Following the hypothesis that tensile force generated by muscle contraction might regulate integrin and IAC turnover during development, I assayed the effect of increasing or decreasing tensile force induced by muscle contraction on integrin and IAC turnover at *Drosophila* MTJs using FRAP. By utilizing several previously described temperature sensitive mutant alleles, I was able to induce muscle hyper-contraction or muscle relaxation by shifting flies from permissive temperatures (PT) to restrictive temperatures (RT). This method allowed me to study integrin and IAC turnover in flies of the same genetic background in a systematic and controlled manner. To increase muscle contraction, I used two sets of temperature sensitive alleles that are known as *Breakdance^{J29} (Brkd^{J29})* and *Swing^{X118} (Swg^{X118})* mutants respectively. At the restrictive temperature (RT) both sets of alleles induce severe and seizure-like muscle contraction (Montana and Littleton, 2004). To decrease muscle contraction, I used *para^{ts2}*, which is a temperature sensitive allele of the *para* gene that encodes a sodium channel in Drosophila that is essential for motor neuron function. At restrictive temperatures (RT), *para^{ts2}* abrogates action potentials in motor neurons, which leads to strong muscle relaxation (Suzuki et al., 1971; O'Dowd et al., 1989; Pittendrigh et al., 1997). I proceeded to test if altering the strength of muscle contraction using *Brkd^{J29}*, *Swg^{X118}*, or *para^{ts2}* mutations can regulate either integrin and/or IAC turnover at MTJs.

3.5.3.2. *Force and IAC turnover*

I investigated the effect of increased muscle contraction on IAC turnover by analyzing the turnover of the IAC marker ILK using FRAP studies in *Brkd*^{J29} and *Swg*^{X118} mutant flies that also express ILK-GFP. These FRAP experiments showed a 62% (p<0.0001, two-tailed *t*-test with Welch corrections) decrease of ILK turnover at embryonic MTJs of *Brkd*^{J29} flies in restrictive temperature (RT) compared to flies kept at permissive temperature (PT)(Fig. 13b,c; Table 6). A similar decrease of 46% in ILK mobile fraction compared to control flies (p=0.0285, two-tailed *t*-test with Welch corrections) was also observed for *Swg*^{X118} flies grown at the restrictive temperature (RT) (Fig. 13a,c; Table 6). The same pattern was also seen at larval MTJs of *Brkd*^{J29} and *Swg*^{X118} flies: I observed decreases of 36% (p=0.0518, two-tailed *t*-test with Welch corrections) and 38% (p=0.0258) respectively compared to control flies kept in permissive temperature (Fig. 14b,c,e; Table 6).

Conversely, I tested whether decreased muscle contraction affects IAC turnover by assaying *para*^{ts2} flies that express ILK GFP. The *para*^{ts2} is well characterized and (Suzuki et al., 1971) and so far temperature sensitive mutant phenotypes have only been described in larvae and adults but not embryos (Suzuki et al., 1971), therefore, I carried out FRAP analysis of IAC turnover in only larval MTJs for *para*^{ts2} flies. My FRAP analysis showed that ILK turnover increased by 88% (p=0.007, two-tailed *t*-test with Welch corrections) at larval MTJs of *para*^{ts2} flies in restrictive temperature compared to control flies kept at room temperature (Fig. 14d,e; Table 6).

Figure 13 – **Increasing tension in embryonic muscles results in down-regulation of ILK turnover:** Whole mount live *Drosophila* embryos with either *Swg* (a,c) or *Brkd* (b,c) allele exhibit lower ILK turnover in restrictive temperatures (RT) than permissive temperatures (PT). Two-tailed t-tests were used to calculate the statistical significance of the differences between mobile fractions of ILK under different levels of tension: (a) *Swg*/ILK-GFP at PT vs RT: n=6, 11; p=0.0285 with Welch correction; (b) *Brkd*/ILK-GFP at PT vs RT: n=21, 18; p<0.0001 with Welch correction. Error bars represent standard error.
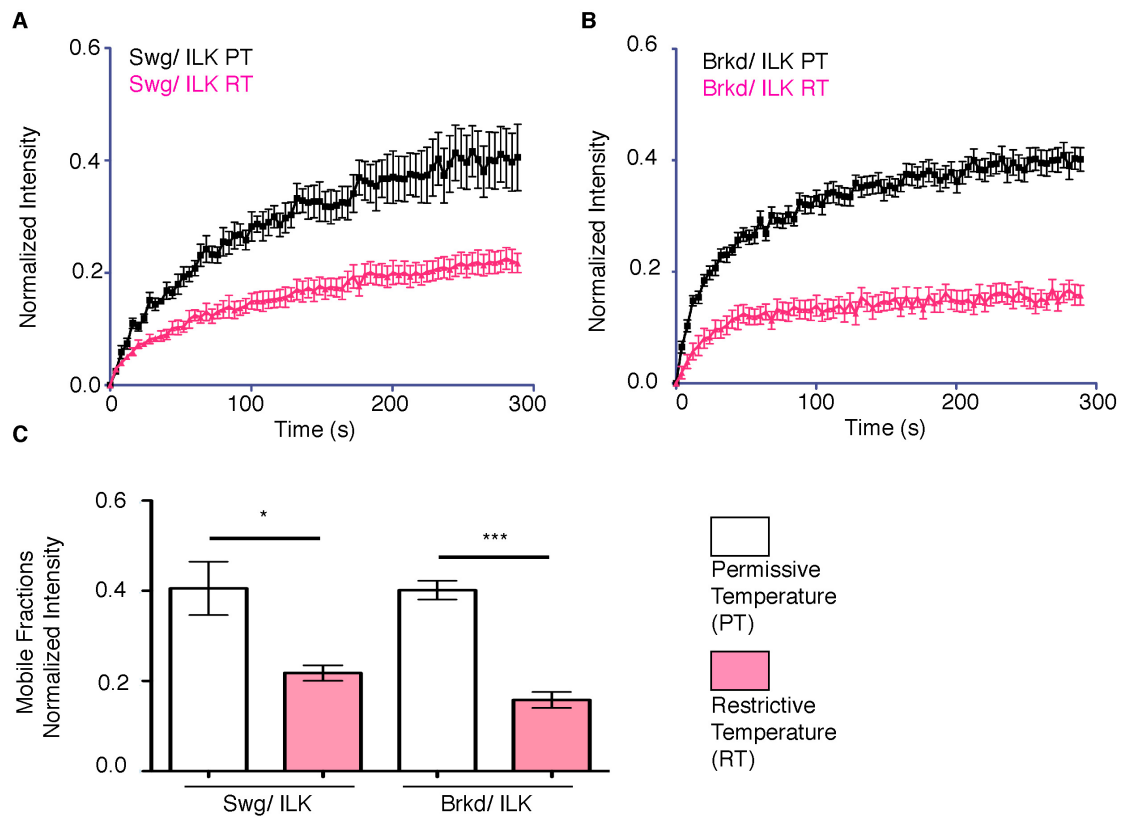
Figure 13 - Increasing tension in embryonic muscles results in down-regulation of ILK turnover

Table 6 - **Mobile fractions of ILK and βPS integrin (wt and Y831FY843F mutant) in response to altered levels of tension**

Genotype	Temp	Stages	MF (Mean±SEM)
Swg/ ILK-GFP	PT	ES 17	0.405±0.059
Swg/ ILK-GFP	RT	ES 17	0.218±0.017
Brkd/ ILK-GFP	PT	ES 17	0.402±0.021
Brkd/ ILK-GFP	RT	ES 17	0.158±0.018
+/ILK-GFP	PT	early L3	0.110±0.015
+/ILK-GFP	RT	early L3	0.100±0.013
Swg/ ILK-GFP	PT	early L3	0.080±0.012
Swg/ ILK-GFP	RT	early L3	0.050±0.004
Brkd/ ILK-GFP	PT	early L3	0.079±0.012
Brkd/ ILK-GFP	RT	early L3	0.050±0.008
Para/ ILK-GFP	PT	early L3	0.090±0.016
Para/ ILK-GFP	RT	early L3	0.170±0.023
+/ βPS integrin-YFP	PT	early L3	0.543±0.015
+/ βPS integrin-YFP	RT	early L3	0.518±0.028
Brkd / βPS integrin-YFP	PT	early L3	0. 581±0.024
Brkd / βPS integrin-YFP	RT	early L3	0.459±0.015
Brkd / βPS integrin-YFP	PT	ES 17	0.621±0.032
Brkd / βPS integrin-YFP	RT	ES 17	0.450±0.028
Para / βPS integrin-YFP	PT	early L3	0.582±0.026
Para / βPS integrin-YFP	RT	early L3	0.561±0.034
Para / βPS integrin*-YFP (Y831FY843F)	PT	early L3	0.502±0.026
Para / βPS integrin*-YFP (Y831FY843F)	RT	early L3	0.616±0.027

Table 6 - Mobile fractions of ILK and integrin in response to altered levels of tension

Figure 14 – **Characterization of ILK turnover at larval MTJs under altered levels of tension:** Whole mount live *Drosophila* larvae with different genetic backgrounds were FRAPed under permissive and restrictive temperatures. Averaged FRAP curves (a) and mobile fractions (e) of +/ILK-GFP under restrictive temperature (RT) and permissive temperatures were the same. Averaged FRAP curves and mobile fractions also showed consistent decreased ILK turnover in hypercontracted muscles induced by placing *Brkd*/ILK-GFP and *Swg*/ILK-GFP flies in restrictive temperatures (RT) (b, c, e) and increased ILK turnover in relaxed muscles induced by placing *Para*/ILK-GFP in restrictive temperatures (RT). Two-tailed t-tests were used to calculate the statistical significance of the differences between mobile fractions of ILK-GFP under different levels of tension: (a) +/ILK-GFP at PT vs RT: n=23, 21; p=0.6352 with Welch correction; (b) *Brkd*/ILK-GFP at PT vs RT: n=16, 13; p=0.0518 with Welch correction; (c) *Swg*/ILK-GFP at PT vs RT: n=18, 16; p=0.0258 with Welch correction; (d) *Para*/ILK-GFP at PT vs RT: n=16, 21; p=0.007 with Welch correction. Error bars represent standard error.
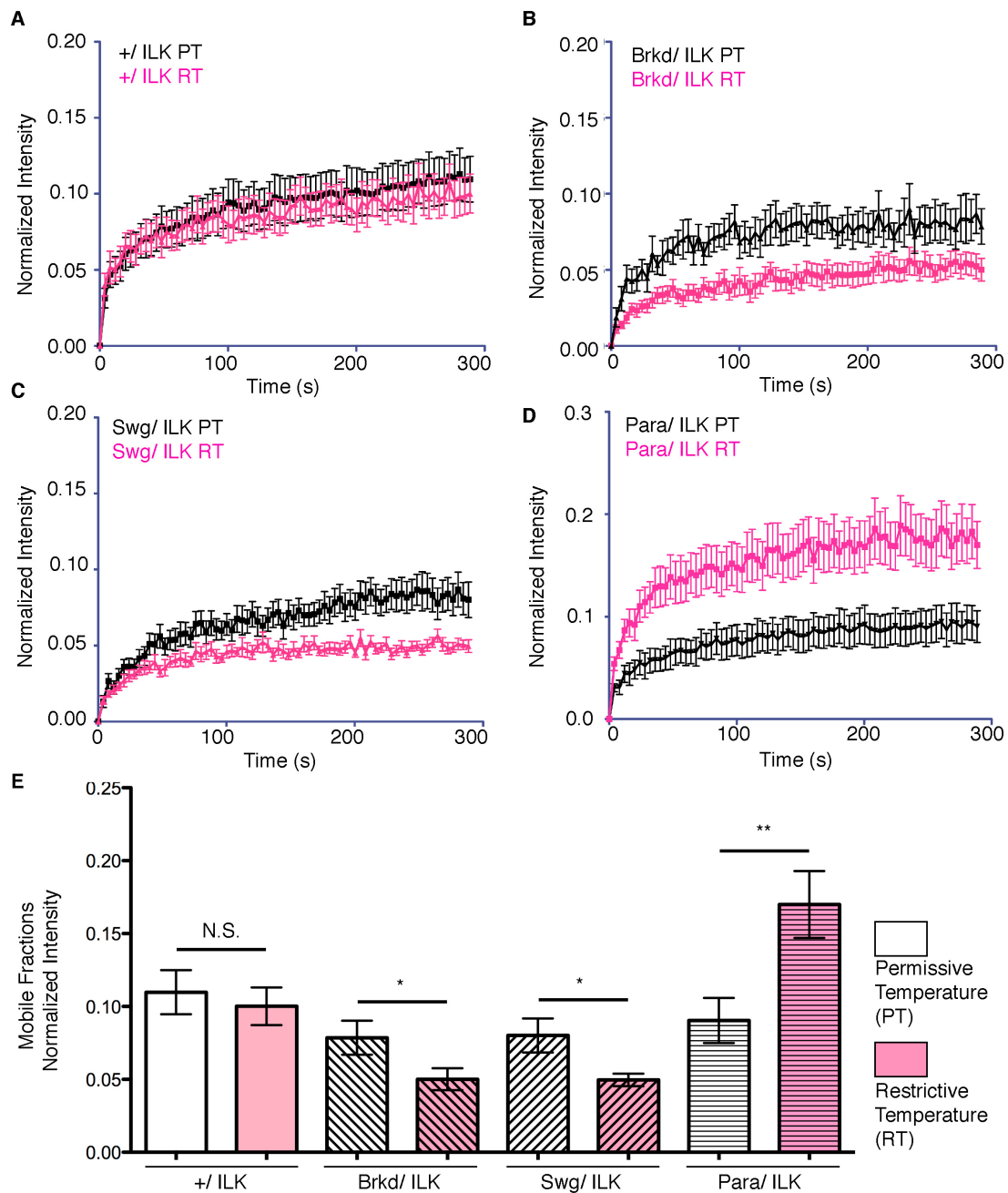
Figure 14 - Characterization of ILK turnover at larval MTJs under altered levels of tension

3.5.3.3.   *Force and integrin turnover*

In order to study whether tensile force can also affect integrin dynamics at MTJs, I employed the same strategy of examining integrin turnover in temperature sensitive muscle contraction mutants at different temperatures. Correspondingly, temperature shift that induced hyper-contraction in *Brkd*[129] flies resulted in a decreased integrin mobile fraction in both embryonic and larval MTJs (embryonic: 27% decrease, p= 0.0005, two-tailed *t*-test with Welch corrections; larval: 21% decrease, p=0.0001; two-tailed *t*-test) (Fig. 15a,c,e; Table 6). However, a reduction in muscle contraction using the *para*[ts2] mutant did not significantly affect integrin turnover (p=0.6252, two-tailed *t*-test) (Fig. 15d,e; Table 6). Because same treatments had always produced similar results on integrin turnover and ILK turnover, I further investigated this inconsistency by using FRAP to study the turnover of an integrin mutant, βPS integrin*-YFP (Y831FY843F). The YY to FF mutation in the cytoplasmic β tail is thought to disrupt phosphorylation of the tyrosines in the two conserved NxxY motifs, an important means of regulating integrin function (Oxley et al., 2008; Anthis et al., 2009b). FRAP analysis showed that βPS integrin*-YFP (Y831FY843F) flies respond to reducing muscle contraction by increasing integrin mobile fractions (23% increase, p=0.0044, two-tailed *t*-test with Welch corrections) (Fig. 15f,g; Table 6).

Figure 15 – **Characterization of integrin turnover under altered levels of tension:** Whether force can also regulate integrin turnover was examined in both live embryos (a) and larvae (b-g). Averaged FRAP curves (a) of +/βPS integrin-YFP under restrictive temperature (RT) and permissive temperature (PT) were the same. *Brkd*/βPS integrin-YFP fly muscles undergo hyper-contraction in the restrictive temperature (RT), which were accompanied with lowered integrin mobile fractions in both embryos (a) and larvae (c) comparing to those in the permissive temperature (PT). Increases in integrin turnover were observed in relaxed muscles induced by placing *Para*/ βPS integrin*-YFP (Y831FY843F) (f) but not *Para*/βPS integrin-YFP (d) in restrictive temperatures (RT). Two-tailed t-tests were used to calculate the statistical significance of the differences between mobile fractions of ILK-GFP under different levels of tension: (a) +/βPS integrin-YFP larvae in PT vs RT: n=21, 20; p=0.4284 with Welch correction; (b) *Brkd*/βPS integrin-YFP larvae in PT vs RT: n=20, 20; p=0.0001; (c) *Para*/βPS integrin-YFP larvae in PT vs RT: n=20, 20; p=0.6252; (d) *Brkd*/βPS integrin-YFP embryos in PT vs RT: n=17, 11; p=0.0005 with Welch correction; (f) *Para*/βPS integrin*-YFP (Y831FY843F) larvae in PT vs RT: n=21, 20; p=0.0044 with Welch correction. Error bars represent standard error.

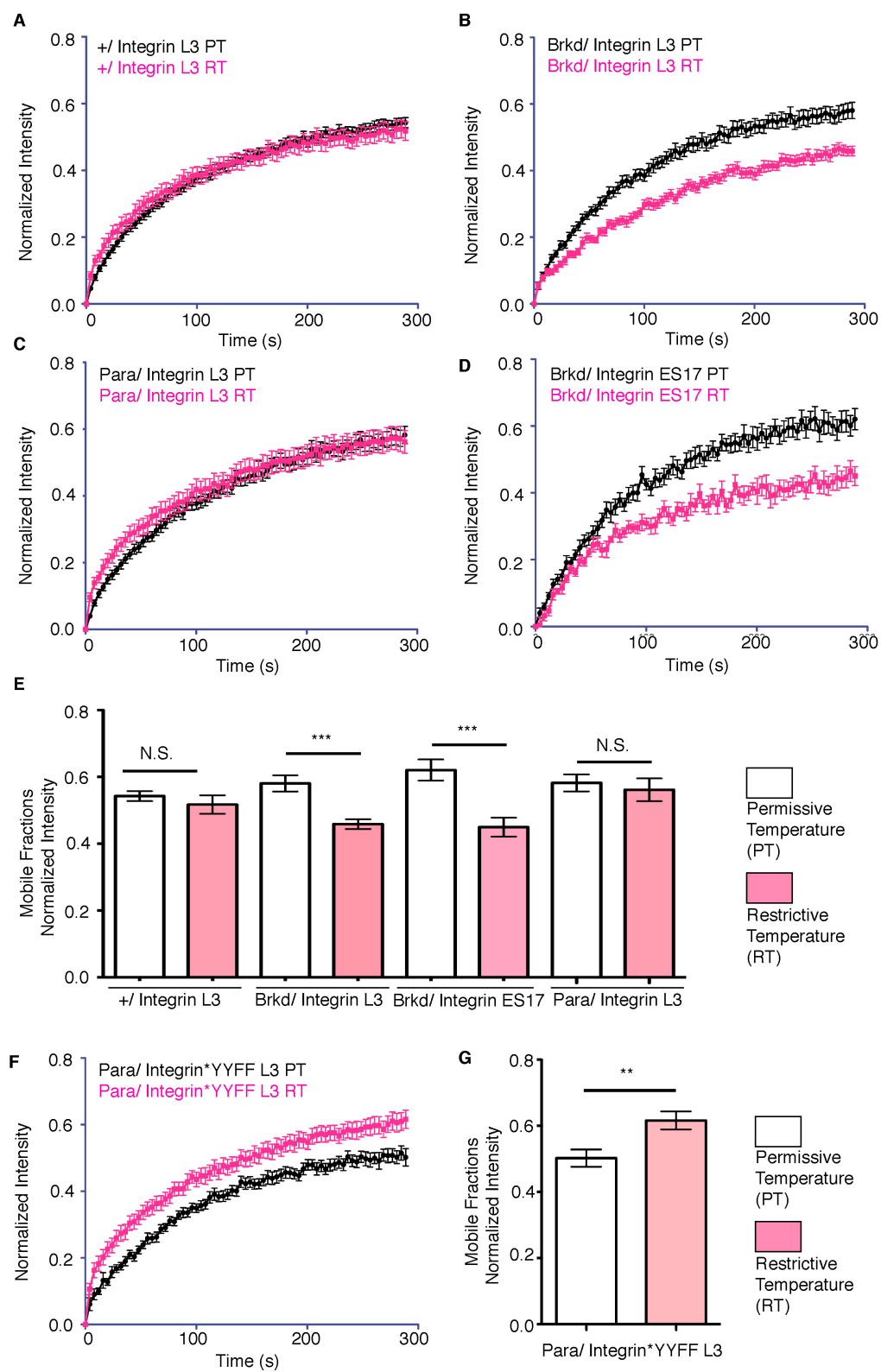**Figure 15 – Characterization of integrin turnover under altered levels of tension**

CHAPTER 4: DISCUSSION AND CONCLUSION

4.1. Integrin-mediated adhesion turnover at MTJs

Experiments outlined in this thesis provide the first study on the turnover of integrin adhesions in live animals. The MTJs analyzed are stable and long-term cell-ECM junctions that form during late embryonic stages and last throughout larval life (about 5 days at room temperature). Though MTJs grow and undergo remodeling at larval stages, they must nonetheless support ongoing muscle attachment during this time. Overall, our results show that components of the IAC at MTJs are highly dynamic. The lowest levels of IAC turnover measured were in 3rd instar larval muscles and even at that stage the mobile fraction of IAC components ranged from as low as 5% for homozygous talin-GFP to as high as 24% for homozygous βPS integrin-YFP.

Surprisingly, I found that a significant proportion of the βPS integrin in MTJs is mobile. Previous studies in cell culture suggested that integrins are mostly immobile within the range of the lifetime of focal contacts (10 to 30 minutes) (Tsuruta et al., 2002; Wolfenson et al., 2009). However, other components of the IAC are highly dynamic and have a half-life of 2-7 minutes (Bretscher, 1989; Bretscher, 1992; Edlund et al., 2001; McKenna et al., 1985; Wolfenson et al., 2009). In the *Drosophila* MTJs, the proportion of βPS integrin that is mobile is in line with other components of the IAC such as talin, tensin and ILK. Although this suggests some differences exist between mechanisms controlling turnover in stable and transient adhesions, I nonetheless found major mechanistic

similarities between turnover in MTJs and focal contacts. For instance, both processes require dynamin-mediated endocytosis and are regulated by the Rab family of small GTPases. This study establishes the MTJ as a useful model to analyze turnover in the context of stable cell-ECM adhesion.

4.2. <u>The contribution of turnover, exchange and diffusion to adhesion dynamics</u>

Mobile fractions of various IAC components were measured to assess their dynamics at MTJs. In the case of integrins, the mobile fraction could be a measurement of turnover (assembly and disassembly) of the IAC, lateral diffusion, and endocytic recycling. FRAP experiments on whole and partial MTJs demonstrates that lateral mobility is not a significant factor contributing to the integrin dynamics measured (Fig 2). For the cytoplasmic components of the IAC the mobile fractions could measure one or more of three processes: "turnover", the assembly and disassembly of the IAC; diffusion of IAC molecules within the cytoplasm; or "exchange", the process in which cytoplasmic IAC components bind and dissociate among the already assembled adhesion complex. For example, a recent study found that the focal adhesion proteins paxillin and vinculin exist in four dynamic states: an immobile focal adhesion-bound fraction, an focal adhesion-associated fraction undergoing exchange, a juxtamembrane fraction undergoing attenuated diffusion, and a fast-diffusing cytoplasmic pool (Wolfenson et al., 2009). Although it is likely that all three processes listed could contribute to the dynamics of various IAC components, I propose that the mobile fraction observed in the MTJ is mainly due to IAC assembly and disassembly rather than diffusion and exchange. This

suggestion is based on two of my observations: Firstly, maximum fluorescence recovery of IAC components following photobleaching was achieved within the range of minutes and seconds rather than milliseconds. Studies in cell culture show that the dynamics of IAC components near the adhesion site are dominated by binding kinetics rather than free diffusion and occur at a similar timescale (Digman et al., 2008; Wolfenson et al., 2009). Secondly, if the mobile fraction of ILK represented only the binding kinetics of ILK with other IAC components, then an increase in the stability of integrin at the MTJ would not reduce the mobile fraction of ILK. However, it was observed that the averaged mobile fractions of both ILK and βPS integrin significantly decline upon inhibition of endocytosis. Nevertheless, it is still possible that ILK undergoes exchange and this might account for some of the 20% of the ILK protein that remained in the mobile fraction when clathrin-mediated endocytosis was inhibited.

4.3. Regulation of IAC turnover and its role in tissue maintenance

Consistent with published results in cell culture showing that other Rab proteins, such as Rab21, regulate integrin-mediated adhesion, the results presented in this thesis show that Rab5 concentrates at MTJs and can regulate the dynamics of IAC molecules at adhesion sites (Pellinen et al., 2006; Tang and Ng, 2009). In migrating cells, overexpression of Rab21 stimulates formation of integrin-mediated cell-ECM adhesion and cell migration while decreased expression of Rab21 impairs cell adhesion and motility (Pellinen et al., 2006; Tang and Ng, 2009). Similarly, I found that the overexpression of Rab5^{DN} and Rab5^{CA} lead to a decrease and an increase of the IAC dynamics respectively (Fig 9). Previous studies conducted in migrating cell also showed that FAK is important for focal

adhesion turnover (Ilic et al., 1995; Owen et al., 1999; Cary et al., 1996; see 1.2.3.). However, reduced autophosphorylation of the *Drosophila* FAK orthologue FAK56D does not affect IAC turnover at MTJs (Appendix B).

Intriguingly, perturbation of IAC turnover via the expression of either Rab5^{DN} or Rab5^{CA} resulted in similar MTJ and muscle defects (Fig 5). It is possible that the increased IAC turnover conferred by Rab5^{CA} expression leads to a reduced capacity for withstanding mechanical tension exerted by muscle contractions. The phenotype induced by Rab5^{DN} expression is somewhat counter intuitive, because reduced IAC turnover would be expected to increase the stability of the IAC. However, it is known that integrins need to constantly respond to environmental cues such as mechanical stress (Ballestrem et al., 2001; Geiger et al., 2009). The Rab5^{DN} phenotype might lead to decreased ability of the IAC to respond to changes in the environment, including mechanical forces, under which the MTJ operates. The hypothesis that distinct mechanisms underlie the Rab5^{CA} and Rab5^{DN} phenotypes could explain the differences in penetrance of the phenotypes conferred by each construct: 27% of Rab5^{CA} expressing embryos and 54% of Rab5^{DN} expressing embryos exhibited muscle defects. Moreover, these findings are consistent with previous work in flies showing that overexpression of integrins gives rise to muscle detachment phenotypes identical to those of integrin null mutants (Tanentzapf et al., 2006). It is not clear why this occurs. Nevertheless, these observations emphasize the importance of precisely regulating the level of Rab5 activity at the MTJ for the maintenance of muscle attachment. It is likely that maintenance of the MTJ necessitates careful regulation of integrin turnover at a precise level. Any deviation from the required

equilibrium between adhesion complex assembly and disassembly leads to muscle detachment.

4.4. Growth-dependent regulation of IAC turnover at MTJs

At the end of muscle morphogenesis (stage 16 of embryogenesis), the IAC in muscles exhibits a high rate of turnover that is similar to that observed in migrating cells. One possible reason for this is that muscle morphogenesis involves highly dynamic processes, such as cell migration and tissue rearrangement, which require extensive IAC turnover. The high levels of turnover observed at the immediate conclusion of muscle morphogenesis may therefore be a lingering after effect of this phase of myogenesis. Another likely explanation is that a certain amount of turnover persists in the newly formed MTJ during embryogenesis to allow the growth and remodelling to take place during larval development. Both possibilities lead to the same prediction that the substantial levels of turnover observed at stage 16 of embryogenesis are generally unsustainable in the more mature larval MTJs. Furthermore, it is possible that a gradual reduction in levels of turnover, similar to our observations in the MTJs, is a general feature of cell adhesion complexes undergoing the transformation from a transient to a stable, long-lasting adhesion.

In addition to stabilizing adhesions, the growth-dependent reduction in the proportion of integrin and IAC components that undergo turnover may play an active role in MTJ growth. Shifting a greater proportion of the integrins in MTJs from the mobile to the

immobile fraction could result in an increase in the size and overall strength of the MTJs so that they can support the strain placed on muscle-tendon attachment as muscles grow (Fig 11 a,b). The question arises as to whether MTJs in adults, which form during pupal stages and last even longer, also exhibit IAC turnover. Adult muscles do not undergo further growth but could potentially undergo remodelling of the MTJs, for example in response to increased mechanical stress. Integrin turnover in the adult might also contribute to repair of MTJs in response to accrued mechanical damage. Due to the presence of an exoskeleton in the adults, it is not currently possible to analyze integrin turnover using FRAP; however, work from our lab has shown that depletion of integrin and other IAC components in adult muscles gives rise to muscle defects, consistent with ongoing adhesion complex turnover (Perkins et al., 2009).

4.5. Mechanotransduction and its role in IAC turnover

The observation that IAC turnover at MTJs is developmentally regulated drove me to search for the underlying mechanism controlling turnover in order to shed light on the more general question of how transient adhesive contacts transform to mature cell junctions during development. To do this, I examined a few candidate regulators. It is possible that signaling pathways known to regulate muscle development, such as Growth Factor Receptor (GFR) pathways and Insulin Receptor (InR) pathways, regulate muscle growth and the growth dependent decrease in integrin and IAC turnover (Yarnitzky et al., 1998; Demontis and Perrimon, 2009). The results presented here suggest that EGFR signaling, a major GFR pathway in *Drosophila*, does not regulate integrin turnover even though EGFR signaling has other important functions in fly muscles, as evidenced by the

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lethality that follows knockdown of EGFR signaling in muscles (Fig. 12). Preliminary analyses on the role of InR signaling in regulating IAC turnover are included in Appendix C.

Alternatively, stage-dependent changes in integrin and IAC turnover could be a secondary consequence of the events surrounding muscle growth. For example, one potential model is that the increasing tension imposed on MTJs by growing muscles could regulate integrin-mediated adhesion turnover. This model is consistent with studies in cell culture that demonstrate a role for intracellular tension mediated by non-muscle actomyosin contraction in focal adhesion formation and cell migration (Vicente-Mansanares et al., 2007; Even-Ram et al., 2007; Choi et al., 2008; Vicente-Manzanares et al., 2009).

Compared to wildtype muscles, hypercontracted muscles exhibited decreased mobile fractions of ILK and integrin (Fig. 8-10). This suggests a role for muscle-specific actomyosin contraction and resulting mechanical tension on adhesion dynamics at cell junctions in live animals. This observation is consistent with published literature showing that force can regulate adhesion complex assembly and function (for details see 1.2.4.). For example, the application of tensile force promotes more stable catch bonds between integrins and ECM ligands and elongates the lifetimes of integrin-ECM bonds in cultured cells (Friedland et al., 2009). Furthermore, stretching of a portion of a talin rod molecule (482-889) *in vitro* unfolds the helical rod and unmasks multiple vinculin binding sites,

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which promotes talin-dependent vinculin activation (del Rio et al., 2009). Activated vinculin mediates binding to the actin cytoskeleton, increases clustering of activated integrins, focal adhesion growth, and mediates force sensing and transmissions at focal adhesions (Le Clainche et al., 2010; Humphries et al., 2007; Dumbauld et al., 2010; Ji et al., 2008). Thus, force has been shown to be an important regulator of adhesion complexes.

In contrast, an increase in ILK turnover was observed in response to decreased tension in relaxed muscles compared to control muscles (Fig. 9). This observation is also consistent with the published literature in the field. Specifically, it was shown that cells exhibit a property called "mechanoreciprocity" meaning that they can turn exogenously applied forces into endogenous tension (Butcher et al., 2009). Decreasing contractile force in fly muscles is similar to softened culture substrates for cultured cells, because both experiments lead to a reduction in the amount of tension that cells experience. Cells grown on flexible, softer, substrates form irregular and more dynamics focal adhesions and show little spreading and high motility (Pelham and Wang, 1997; Yeung et al., 2005).

Although the application of mechanical tension led to down-regulation in the dynamics of both ILK and integrin, I found that increased muscle relaxation up-regulated ILK (p=0.007) but not integrin turnover (p=0.6252) (Fig. 9,10). This kind of qualitatively different response, supported by robust statistical analyses, was never observed in other

experiments, suggesting that ILK and integrin turnover are regulated differently in relaxed muscles.

It is possible that the turnover of cytoplasmic IAC proteins, like ILK, but not integrin turnover is modulated in response to decreased mechanical tension. Alternatively, a general mechanism exists that increases IAC turnover in response to lower mechanical tension but that integrins are somehow exempt from this regulatory mechanism. I tested this second possibility by studying the turnover of a fluorescently labeled βPS integrin that contains a mutation known to affect the regulation of integrins by abolishing two key known phosphorylation sites in the integrin (pUbi βPS integrin*-YFP (Y831FY843F)). Tyrosine phosphorylations of the two conserved NxxY motifs on β integrin tail can influence integrin signaling in both directions but have cell-specific and integrin-specific effects (Pylayeva and Giancotti, 2006). My preliminary results indicate that YFP tagged integrin containing this YY to FF mutation undergoes a similar increase in mobile fraction when muscles were relaxed, similar to that observed with ILK (Fig. 9, 10). This indicates that phosphorylations of tyrosines in the integrin cytoplasmic tail may act to inhibit an increase in turnover in response to decreased tension.

It is not immediately clear why the YY to FF mutation would result in increased integrin turnover in response to reduced tension. Y to F mutations in the vertebrate β1, β3 and β7 integrins enhance integrin-talin interactions and reduce interactions with other proteins that bind to the integrin cytoplasmic tail such as Src family kinases (Wennerburg et al.,

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2000; Oxley et al., 2008; Anthis et al., 2009b). Moreover, YY to FF mutations of the two conserved NxxY motifs inhibits the internalization of integrins through clathrin-mediated but not Rab21-mediated pathways in cultured cells (Pellinen et al., 2008). Future work to elucidate how integrin turnover is regulated by force at MTJs using point mutated YFP tagged integrins is already in progress. This work should shed more light on the mechanisms that selectively regulate integrin turnover in response to mechanical stress.

The question arises as to why a mechanism that inhibits increased integrin turnover in response to reduced stress would exist. One possibility could be that increased integrin turnover when cells are exposed to less mechanical strain is not desirable. It takes a great deal of energy to produce and localize integrin to sites of integrin-mediated adhesion. If integrins underwent increased turnover every time the cell was exposed to lowered mechanical strain, this could be energetically expensive for the cell. Moreover, integrins undergoing higher turnover may carry substantial risk to the cell, because it leaves the junction in a weakened state, which is more likely to fail, if and when external mechanical forces resume. Additionally, muscle cells may need to maintain a certain level of activated integrins on the cell membrane to mediate transduction of growth and survival signals in a similar way as noncancerous cells cannot survive on a soft surface due to decreased outside-in signaling and integrin activation (Georges and Janmey, 2005). Therefore, there may be a number of advantages to the cell in preventing increased integrin turnover in response to reduced mechanical stress.

4.6. Conclusions

Studies described in this thesis demonstrate that stable, long-term IACs in MTJs are dynamic. Integrin and IAC turnover requires clathrin-mediated endocytosis of integrins from the plasma membrane. Consistent with the known role of Rab GTPases in regulating integrin turnover in migrating cells, Rab5 was found to regulate IAC turnover and, consequently, the integrity of MTJs *in vivo*. Moreover, the growth of MTJs that occurred as *Drosophila* larvae develop correlated with decreases in the dynamics of several IAC components. Finally, increasing levels of mechanical stress exerted by muscle hyper-contraction down-regulated both integrin and IAC turnover at MTJs, while reduced tension up-regulated IAC turnover at MTJs. Furthermore, up-regulation of integrin dynamics may be inhibited by tyrosine phosphorylation of the β integrin tail. Therefore, the turnover of stable, long-term adhesions is a coordinated process of IAC disassembly, which can be influenced by clathrin-mediated endocytosis, Rab5-mediated recycling of IAC components through intracellular trafficking, activation of integrins and IAC assembly (Ezratty et al., 2005; Ezratty et al., 2009; Shattil et al., 2010; Caswell et al., 2009). Biophysical and biochemical factors provide regulatory cues that control the steps that underlie the turnover of integrin-mediated adhesion. These results illustrate how the precise regulation of adhesion turnover is required to maintain tissue integrity throughout development.

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APPENDICES

Appendix A - The C-terminal talin Actin Binding Domain (ABD) is functionally conserved in Drosophila.

(A-C) Muscles in wildtype, live, stage 17 embryos expressing the GFP-tagged C-terminal talin Actin Binding Domain (ABD) (WT-ABDGFP; A) and mutated versions of talin-ABD that disrupt actin binding (KVK/DDD-ABD-GFP; B) or dimerization (R2531G-ABD-GFP; C). (D) Muscles in zygotic rhea mutants in live, stage 17 embryos expressing the GFP-tagged C-terminal talin-ABD. (E) Enrichment of wildtype and mutant GFP tagged talin-ABD transgenes at muscle termini expressed as a ratio of averaged fluorescence intensity at the attachment sites to cytoplasmic staining (*, $p < 0.05$; **, $p < 0.001$ in a two-tailed t-test). (F-H) Muscles stage 17 embryos expressing wildtype and mutant versions of talin-ABD co-stained with Rhodamine-Phalloidin to label actin. GFP fluorescence appears weaker due to formaldehyde fixation.
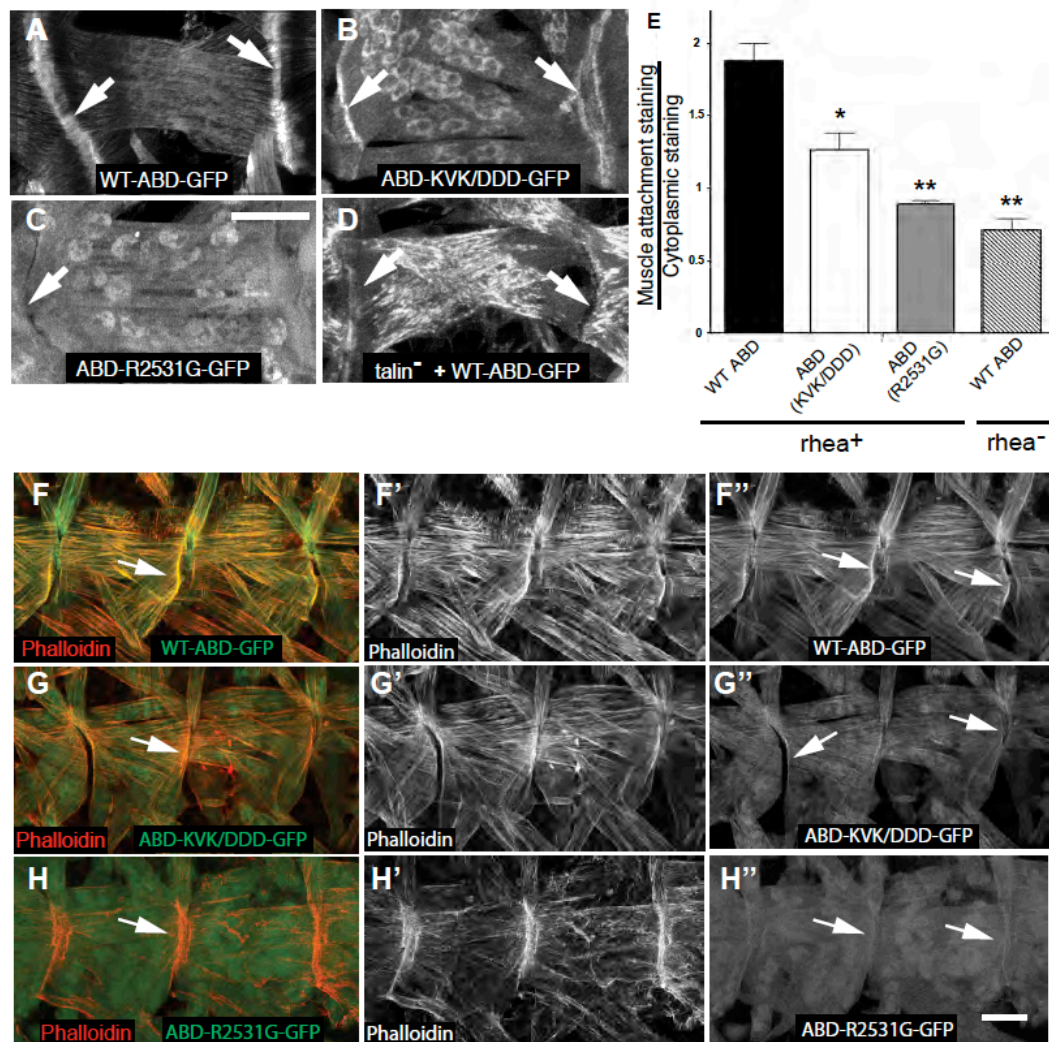
Appendix B - Reduced autophosphorylation of *Drosophila* FAK does not affect IAC turnover at MTJs.

The tyrosine 430 residue in *Drosophila* FAK56D is equivalent to the tyrosine 397 residue in vertebrate FAK and both are major autophosphorylation sites for FAK functions (Fujimoto et al., 1999). When FAK Y430F is expressed in *Drosophila* muscles using the UAS-Gal4 system, changes in ILK turnover were found neither in embryos (p=6898; two-tailed t test with Welch corrections)(A,C) nor in larvae(p=0.8167; two-tailed t test with Welch corrections) (B,C). This is consistent with research showed FAK is not required for integrin function or viability in *Drosophila* (Grabbe et al., 2004). Nevertheless, Y430F point mutation in FAK does not abolish all of the autophosphorylation but reduce it by half (Fujimoto et al., 1999). Therefore, future experiments can be carried out using a K513M point mutation in *Drosophila* FAK56D, which abolishes all autophosphorylation of FAK56D (Fujimoto et al., 1999). Moreover, because overexpression of FAK ubiquitously leads to lethality in *Drosophila*, it would be interesting to study if overexpressing FAK will perturb IAC turnover in the future (Grabbe et al., 2004).
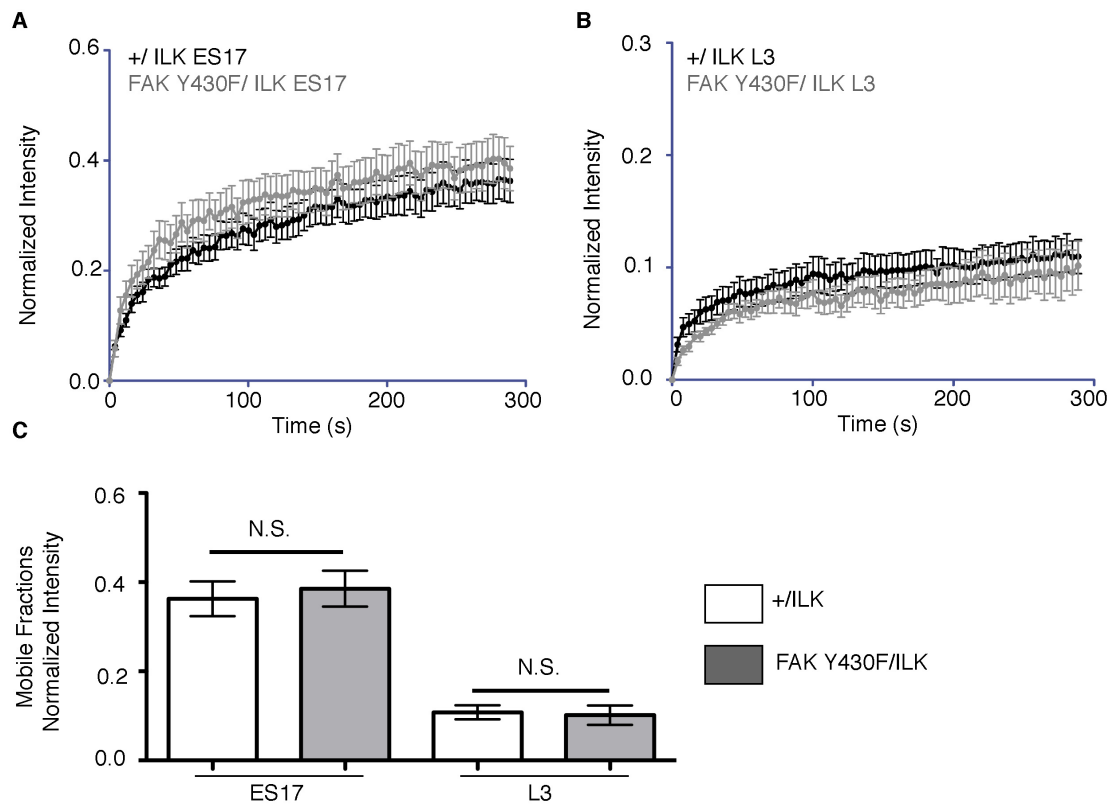
Appendix C - Characterization of the role of Insulin Receptor (InR) signaling pathway in IAC turnover.

(A) Up-regulation of the InR pathway by overexpressing InR-CA in muscles resulted in a decrease in ILK turnover at embryonic MTJs (p=0.0011; two-tailed t test), while (B,C) down-regulation of the InR pathway by overexpressing InR-DN (p=0.1382; two-tailed t test) or foxo (t=0.1718; two-tailed t test) in muscles does not change the ILK turnover at embryonic MTJs. However, neither up-regulation nor down-regulation results in a change in ILK dynamics at larval MTJs (InR-CA: p=0.1608; InR-DN: p=0.5239; foxo: p=0.9992 two-tailed t tests with Welch corrections) (D-F).