Analysis of integrin turnover in fly myotendinous junctions

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Summary

Transient (short-term) cell adhesion underlies dynamic processes such as cell migration, whereas stable (long-term) cell adhesion maintains tissue architecture. Ongoing adhesion complex turnover is essential for transient cell adhesion, but it is not known whether turnover is also required for maintenance of long-term adhesion. We used fluorescence recovery after photobleaching to analyze the dynamics of an integrin adhesion complex (IAC) in a model of long-term cell-ECM adhesion, myotendinous junctions (MTJs), in fly embryos and larvae. We found that the IAC undergoes turnover in MTJs and that this process is mediated by clathrin-dependent endocytosis. Moreover, the small GTPase Rab5 can regulate the proportion of IAC components that undergo turnover. Also, altering Rab5 activity weakened MTJs, resulting in muscle defects. In addition, growth of MTJs was concomitant with a decrease in the proportion of IAC components undergoing turnover. We propose that IAC turnover is tightly regulated in long-term cell-ECM adhesions to allow normal tissue growth and maintenance.

Key words: *Drosophila*, Integrin, Turnover, Muscle, Cell adhesion, Myotendinous junctions, Endocytosis, Rab5

Introduction

Cells in multicellular organisms are arranged in highly complex three-dimensional patterns. Achieving such a complex organization necessitates that cells form both transient (short-term) and stable (long-term) adhesive contacts with each other (cell-cell adhesion) and with their extracellular environment (cell adhesion to the ECM or cell-ECM adhesion). Adhesion complex turnover, a process in which adhesion complexes assemble and disassemble at the cell surface, plays an important role in regulating the strength and duration of cell adhesion (Ulrich and Heisenberg, 2009; Webb et al., 2002). A decrease in the turnover rate of adhesion complexes might lead to the formation of stable adhesion, whereas an increase in turnover rate might lead to more transient adhesive contacts.

The integrin family of adhesion receptors can mediate both transient and stable cell-ECM adhesion. Integrin heterodimers, composed of an α- and a β-integrin subunit, bind ECM proteins and, at the same time, assemble a large, intracellular multiprotein complex. The assembly of these integrin adhesion complexes (IACs) is orchestrated mainly through interactions of the cytoplasmic tail of the β-integrin subunit (Liu et al., 2000). The function of the adhesion complex that assembles around this short cytoplasmic tail is twofold: to regulate the binding of integrins to the ECM and to link integrins to the cytoskeleton.

Transient integrin-mediated adhesion to the ECM is known to play an important role during cell migration (Webb, 2003). Studies in cell culture have shown that integrins in migrating cells are highly dynamic and transmembrane integrins are constantly endocytosed (Bretscher, 1989; Bretscher, 1992). Furthermore, the purpose of this internalization is to recycle rather than degrade integrins (Bretscher, 1989; Bretscher, 1992). Recent work has led to the emergence of a model whereby integrin-mediated adhesion undergoes turnover through a cycle of adhesion complex disassembly and internalization, followed by recycling of integrins to the membrane and adhesion complex assembly. This manner of adhesion complex turnover is essential for cell migration (Caswell and Norman, 2006; Pellinen and Ivaska, 2006). For example, blockage of dynamin-mediated endocytosis in cell culture leads to failure in the disassembly of focal adhesions (FAs) and disruption of cell migration (Ezratty et al., 2005).

The Rab family of small GTPases is implicated in regulating integrin recycling during cell migration (Caswell et al., 2008; Caswell and Norman, 2006; Caswell et al., 2007; Pellinen et al., 2006; Pellinen and Ivaska, 2006; Tang and Ng, 2009). Rab GTPases have established roles in tethering and fusion of membrane vesicles, as well as in transport of vesicles and associated cargo proteins (Zerial and McBride, 2001). Various Rab proteins, including Rab4, Rab5, Rab11, Rab21 and Rab25, have essential roles in regulating the endosomal traffic and subcellular localization of integrins during cell spreading and cell migration (Pellinen and Ivaska, 2006; Roberts et al., 2001; Tang and Ng, 2009). It has been proposed that the ability of regulators of endocytosis, such as Rab proteins, to rapidly modulate IAC turnover gives cells the potential to exert rapid control over integrin function, which is advantageous in the context of cell migration (Caswell and Norman, 2006).

In addition to mediating transient adhesion, integrins are important for the stable cell-ECM adhesion that maintains tissue structure. For example, the formation and long-term maintenance of muscle-tendon attachment at myotendinous junctions (MTJs) requires integrin-mediated adhesion in humans (Hayashi et al., 1998), mice (Mayer et al., 1997) and flies (Brown et al., 2000). Fly MTJs form at the end of embryogenesis and persist throughout the larval stages (Bate, 1993). In the absence of integrin-mediated adhesion, the initial specification, fusion and attachment of the muscles proceeds normally, but shortly after the muscles form they detach and round up (Brown, 1993).

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To date, the role of integrin turnover in maintaining stable tissue architecture over long periods of time has not been addressed, mainly because of the difficulty of analyzing adhesion complex turnover in live animals. However, studies in rat skeletal muscle fibers showed extensive endocytic activity at the MTJ (Kaisto et al., 1999), suggesting that integrins could be undergoing turnover at these sites. Here, we present our analysis of integrin and IAC turnover in fly MTJs in intact live animals. Our analysis reveals that MTJ turnover is mediated by the endocytic machinery and plays an important role in muscle growth and maintenance.

Results

IAC components undergo turnover at MTJs

We asked whether, once assembled, the IAC at the MTJs undergoes turnover. To visualize the turnover of components of the IAC, we used fluorescently tagged constructs of β position-specific (βPS) integrin (βPS-integrin-YFP) and of the core structural IAC components talin (talin-GFP) and tensin (tensin-GFP) (see Materials and Methods), as well as a viable line with a GFP inserted in the genomic *ilk* (*integrin-linked kinase*) gene (ILK-GFP) (Morin et al., 2001). All fluorescently tagged constructs rescued the embryonic phenotypes of null mutations in their respective genes and faithfully reproduced the expression pattern of the endogenous untagged molecule (Hudson et al., 2008; Torgler et al., 2004) (G.T., data not shown).

Fluorescence recovery after photobleaching (FRAP) allows the measurement of the mobile fraction, which represents the amount of mobile molecules as a proportion of all fluorescent molecules present in the membrane. We used FRAP to determine the mobile fraction of integrin and the core components of the IAC. The MTJ in fly embryos and larvae is especially suitable for FRAP analysis, because it forms a distinct, thin, linear adhesion site at the ends of the muscle (Fig. 1A). FRAP experiments on homozygous integrin-YFP flies revealed that, in mature MTJs of late-stage embryos, the mobile fraction of βPS-integrin was 40% (Fig. 1B). We also computed the $t_{1/2}$ values for all the FRAP experiments we carried out, but found this measurement to be inconclusive because the standard error values were very large (supplementary material Table S1).

Because integrin is a transmembrane protein, the measured mobile fraction of βPS-integrin could represent either lateral diffusion of integrins across the plasma membrane or integrin turnover. To determine whether the observed mobile fraction was due to lateral diffusion, we compared experiments in which the integrin-YFP of an entire MTJ was photobleached with experiments in which only a small section of the integrin-YFP of MTJ was photobleached (Fig. 2). 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. This is consistent with previously published results showing that integrins have low lateral mobility in stable adhesive contacts (Duband et al., 1988) and indicates that the mobile fraction of βPS-integrin in the MTJ is a measure of turnover. It should be noted that successful photobleaching of an entire muscle attachment requires adjustment of the FRAP settings (see Materials and Methods). These adjustments led to the observation of different values for the mobile fraction than FRAP experiments using homozygous integrin-YFP flies (compare to Fig. 1B). FRAP experiments on tensin, talin and ILK also revealed the presence of a mobile fraction (Fig. 1C-E). The $t_{1/2}$ values for all IAC components studied were less than 100 seconds and no recovery occurred more than 300 seconds after photobleaching (supplementary material Table S1).

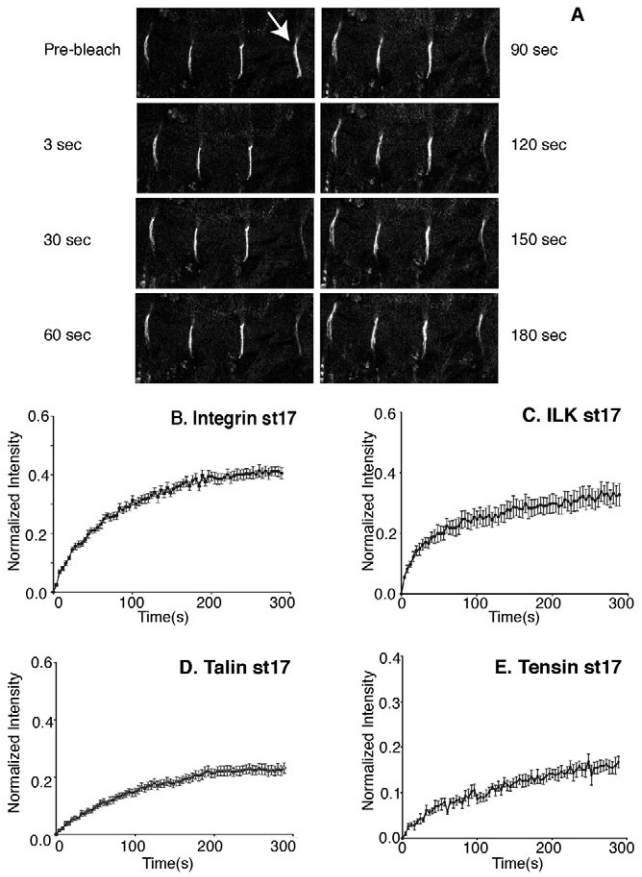

Fig. 1. Integrin, talin, ILK and tensin undergo turnover in MTJs. (**A**) Time course of recovery of integrin-YFP after photobleaching one muscle attachment (arrow) in a stage 17 embryo. The other muscle attachments are shown as controls. (**B-E**) Averaged FRAP recovery curves for stage 17 embryo integrin-YFP (B), ILK-GFP (C), talin-GFP (D) and tensin-GFP (E). Each recovery curve is an average of ten individual FRAP experiments; error bars represent standard error.

IAC turnover requires clathrin-mediated endocytosis

To explore the mechanisms underlying integrin turnover in MTJs, a mutant temperature-sensitive allele of dynamin (upstream activating sequence-*shibere*^{ts}; UAS-*shi*^{ts}) (Kitamoto, 2001) was used to inhibit endocytosis in fly muscles. We expressed UAS-*shi*^{ts} and used high temperature to reduce dynamin activity in muscles (see Materials and Methods). We found that our control heterozygous integrin-YFP flies exhibited a slightly higher fluorescence recovery in larval myotendinous junctions after photobleaching under the high-temperature conditions compared with at room temperature. Otherwise, the FRAP curves were unaffected. The muscle-specific expression of *shi*^{ts} led to a small but statistically significant reduction in the mobile fraction of βPS-integrin and ILK (Fig. 3A,B; Table 1). The small effect conferred by UAS-*shi*^{ts} is probably the result of the limitations of using an ectopic, temperature-sensitive construct in an otherwise wild-type background. To confirm the role of endocytosis in integrin turnover, we took a pharmacological approach using the dynamin inhibitor dynasore (Macia et al., 2006). A protocol was devised to enable live imaging of dissected flat

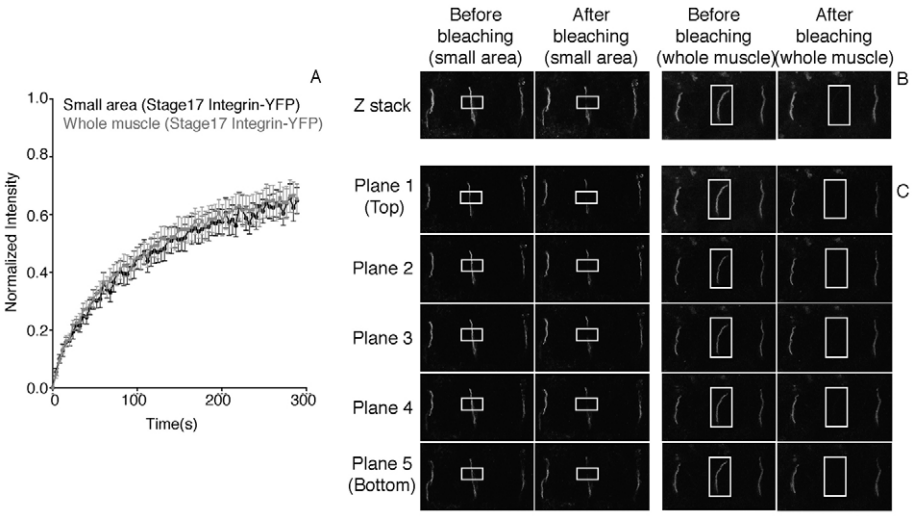

Fig. 2. Integrins do not exhibit lateral mobility. (**A**,**B**) Averaged FRAP recovery curves for integrin-GFP in intact live 3rd instar larva are nearly identical (n=12, p=0.9462, two-tailed t-test) when the entire MTJ is photobleached or when only a small section of the junction is photobleached. (**C**) As *Drosophila* embryonic MTJs are typically thin, photobleaching occurs efficiently in each plane of the MTJ. The total Z-stack thickness is 3 μm, with each focal plane 0.6 μm apart.

preparations of 3rd instar larval muscles and epidermis containing intact MTJs (see Materials and Methods). These flat preparations exhibited normal muscle contraction for a few hours following dissection and mounting, and the fluorescent intensity of both ILK-GFP and integrin-YFP recovered following photobleaching. We did notice that the mobile fractions observed in controlled (untreated) MTJs were lower for integrin-YFP and higher for ILK-GFP when compared to that seen in intact larvae of the same genotype (Fig. 3C,D). This is probably due to the fact that these experiments necessitated the use of different FRAP settings (see Materials and Methods), but might reflect other innate differences between culture conditions and in vivo conditions. When 3rd instar larval flat preparations were treated with dynasore, a small cell-permeable molecule that acts as a potent inhibitor of dynamin (Macia et al., 2006), the mobile fractions of ILK and βPS-integrin were reduced by 63% and 50%, respectively (Fig. 3C,D). Because dynamin can mediate both clathrin-dependent and clathrin-independent endocytosis (Caswell and Norman, 2006; Pellinen and Ivaska, 2006), we used chlorpromazine (CPZ), which is used to specifically inhibit clathrin-dependent endocytosis in both cell culture systems

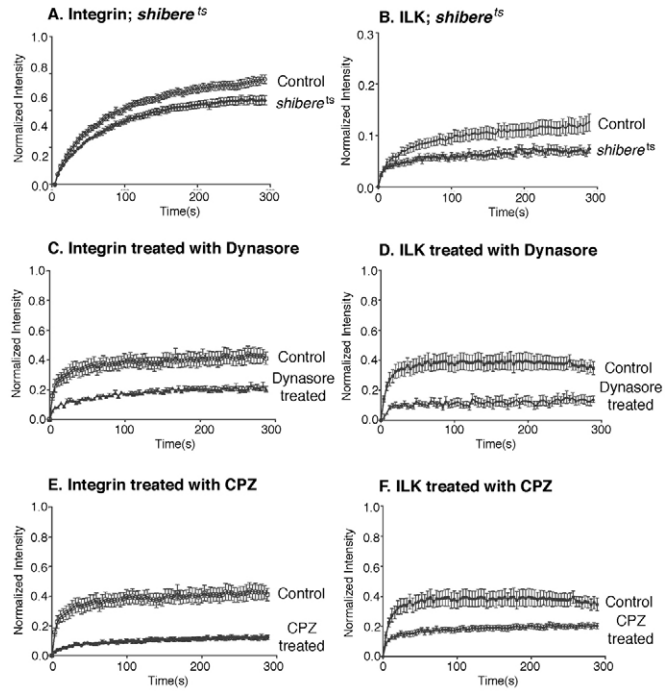

Fig. 3. Integrin turnover at the MTJ requires clathrin-mediated endocytosis. (**A**,**B**) Averaged FRAP recovery curves in intact live 3rd instar larva for integrin-YFP/+ (A) and ILK-GFP/+ (B) in a wild-type background (control) and in muscles expressing a UAS-*shi*^{ts} transgene. (**C-F**) Averaged FRAP recovery curves for integrin-YFP (C,E) and ILK-GFP (D,F) in a 3rd instar wild-type larval flat preparation with or without treatment with dynasore (C,D) or CPZ (E,F). Statistical analysis of the significance of differences between averaged FRAP curves is 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.

Table 1. FRAP analysis of mobile fraction for βPS-integrin–YFP and ILK-GFP flies expressing *shi*^{ts}, Rab5-DN or Rab5-CA, or treated with dynasore or CPZ

	MF (L3) (mean ± s.e.m.)
Genotype	
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
Larval flat preparation	
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

*Experiments with UAS-*shi*^{ts} were carried out at higher temperature (see Materials and Methods); at these temperatures, integrin-YFP and ILK-GFP recoveries are slightly higher.

L3, 3rd instar larvae; MF, mobile fraction.

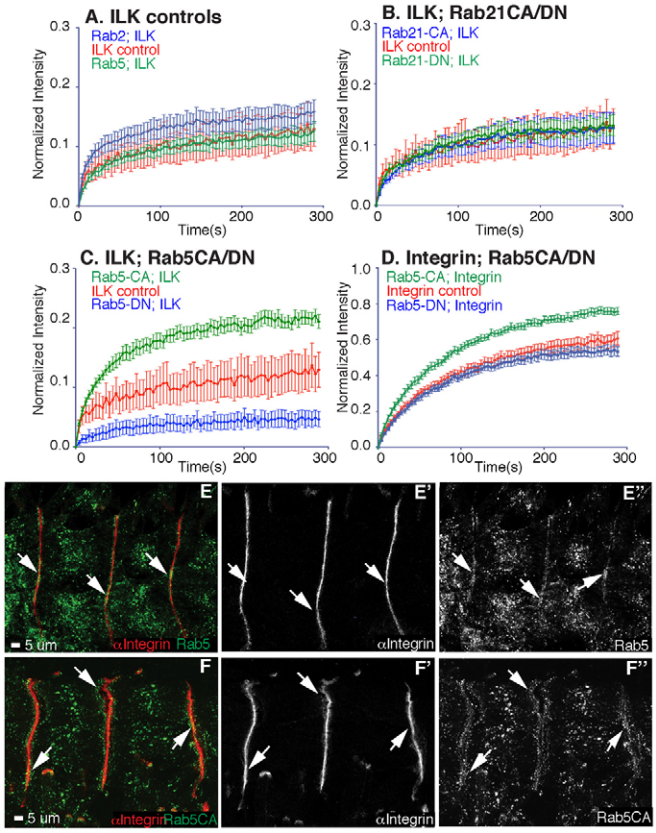

Fig. 4. Characterization of the effects of Rab5-CA and Rab5-DN on IAC turnover. (**A-D**) Averaged FRAP recovery curves in intact live 3rd instar larva for ILK-GFP/+ (A-C) and integrin-YFP/+ (D) in a wild-type background and in muscles expressing Rab transgenes. (A) Expression of Rab2 (as a control) or Rab5 did not affect the recovery of ILK-GFP. (B) Expression of CA or DN Rab21 does not change the recovery of ILK-GFP following photobleaching. (C,D) Expression of CA or 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 the significance of differences between averaged FRAP curves is by one-way ANOVA test: (A) n=10, p=0.4721; (B) n=13, p=0.4054; (C) n=10, p<0.0001; (D) n=18, p<0.0001. One-tailed t-test: (C) ILK versus Rab5CA, ILK: p=0.0054; ILK versus Rab5DN, ILK: p=0.0082; (D) integrin versus Rab5CA, integrin: p=0.0008; integrin versus Rab5DN, integrin: p=0.07. Error bars represent standard error.

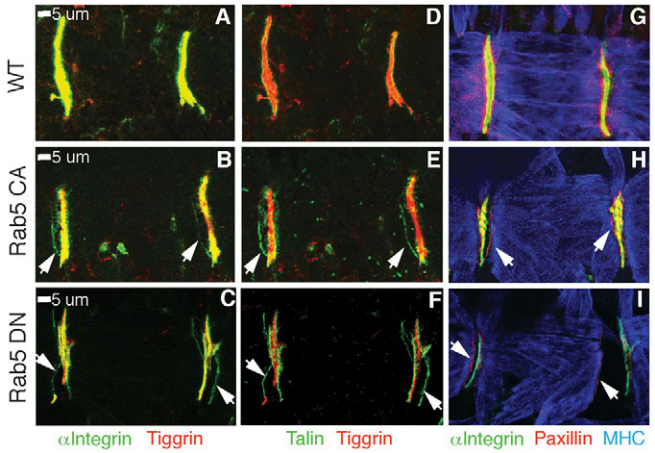

Fig. 5. Expression of Rab5-CA and Rab5-DN in muscles induces detachment of integrins from the ECM. (**A**) In wild-type muscle, αPS2 integrin (green) overlaps with the ECM (marked with tiggrin, red). (**B**,**C**) Expression of Rab5-CA (B) or Rab5-DN (C) led to separation of integrin and the ECM (arrowheads). (**D**) The ends of the muscle, marked with talin (green), are in contact with the ECM (marked with tiggrin, red) in wild-type muscles. (**E**,**F**) Expression of Rab5-CA (E) or Rab5-DN (F) led to separation of muscle ends and the ECM (arrowheads). (**G**) The IAC protein paxillin (red) colocalizes with αPS2 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. Scale bars: 5 μm.

and *Drosophila* larvae (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. 3E,F). These results indicate that integrin turnover in stable adhesions requires active clathrin-dependent endocytosis of integrins from the plasma membrane.

Rab GTPases regulate IAC turnover

The Rab family of GTPases has been implicated in regulating integrin turnover in migrating cells; we therefore asked whether they were involved in regulating integrin turnover in the MTJ. Specifically, we tested the effects of dominant-negative and constitutively active versions of Rab21 (Rab21-DN and Rab21-CA) and Rab5 (Rab5-DN and Rab5-CA) in fly muscles (Zhang et al., 2007). Muscle-specific expression of neither Rab21-DN nor Rab21-CA altered the ILK dynamics at MTJs (Fig. 4B; Table 1). However, a small but statistically significant 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. 4C,D; Table 1). This is consistent with the role of Rab5 in IAC turnover described previously (Pellinen et al., 2006). Furthermore, a population of Rab5-positive vesicles were observed to concentrate near the MTJ and overlap with integrin expression in embryonic muscles (Fig. 4E,F). The activation of Rab5 has been shown to increase the motility of endosomes along microtubules (Dinneen and Ceresa, 2004; Nielsen et al., 1999). Expression of a constitutively active Rab5 (Rab5-CA) in fly muscles conferred an increase in the mobile fractions of βPS-integrin and ILK of 25% and 62%, respectively, suggesting that Rab5 activation also promotes IAC turnover at stable adhesions (Fig. 4C,D; Table 1). In comparison, expression of a wild-type version of Rab5 or the secretory regulator Rab2 did not change the mobile fraction of ILK (Fig. 4A; Table 1).

Inhibiting integrin turnover in migrating cells stalled cell migration (Woods et al., 2004; White et al., 2007). We therefore analyzed the effect of altering integrin turnover, using Rab5-CA and Rab5-DN, on muscle attachments. We found that expressing either Rab5-CA (Fig. 5B,E,H) or Rab5-DN (Fig. 5C,F,I) induced muscle-detachment defects in some muscles when compared with the wild type (Fig. 5A,D,G). Using whole-embryo confocal image stacks to identify detached muscles showed 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 failure of IAC assembly, but rather to separation

between the integrins at the end of the muscles and the ECM (Fig. 5B,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). In addition, wider and more diffuse integrin expression was observed in muscles expressing Rab5-CA (Fig. 4F). These results show that Rab5 regulates the strength and shape of the MTJs, and that regulation of integrin turnover is essential for maintaining MTJ integrity. Moreover, because MTJ-attachment defects could potentially affect the mobile fraction, all the FRAP experiments with Rab5-CA and Rab5-DN were carried out in intact muscles.

IAC turnover at MTJs is developmentally regulated

During *Drosophila* larval stages, the muscles undergo dramatic growth that is accompanied by an increase in MTJ width by a factor of 4 (Fig. 6A). 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 larva are not well understood, but we hypothesize that they involve a change in the dynamics of integrin turnover. Interestingly, we found that there is a stage-dependent decrease in the mobile fraction of IAC components following MTJ formation (Fig. 6B-E). This decline begins during embryogenesis; 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. This trend continues such 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. Nonetheless even in 3rd instar larva, a persistent low level of IAC turnover at the MTJ, as high as 24% in the case of βPS-integrin, remains.

Discussion

We provide here the first study of the turnover of integrin adhesions in live animals. The MTJs analyzed are long-lasting cell-ECM adhesions that form during late embryonic stages and last throughout larval life (about 5 days at room temperature). Although MTJs grow and undergo remodeling at larval stages, they must nonetheless support ongoing muscle attachment during this time. Overall, our results show considerable IAC dynamics in the MTJs. 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 integrin-YFP.

Surprisingly, we 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 life-time of focal contacts (10 to 30 minutes) (Tsuruta et al., 2002; Wolfenson et al., 2009), whereas other components of the IAC are highly dynamic and have a half-life on the order of 2-7 minutes (Bretscher, 1989; Bretscher, 1992; Edlund et al., 2001; McKenna et al., 1985; Wolfenson et al., 2009). In the 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 that some differences exist between the turnover mechanisms of stable and transient adhesions, we nonetheless found major mechanistic similarity 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.

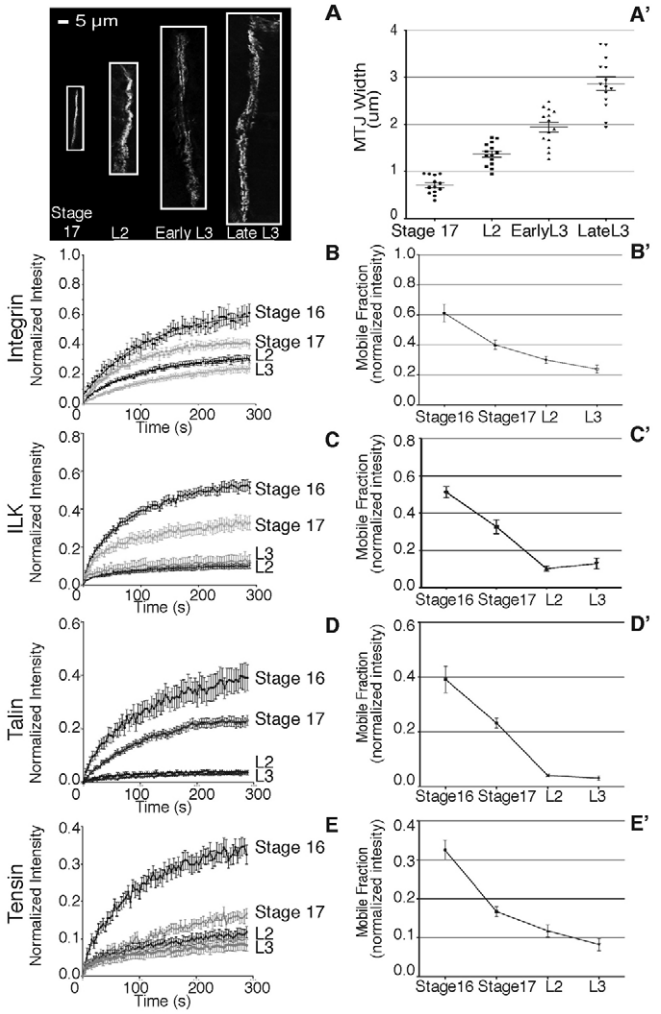

Fig. 6. Stage-specific reduction in IAC mobility in the MTJ. (**A**,**A′**) Quantification of MTJ width at progressive stages of larval development; MTJ width increases by a factor of four on average during this time. Average FRAP recovery curves (**B-E**) and corresponding mobile fraction values (**B′-E′**) for 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. All error bars represent standard error.

The contribution of turnover, exchange and diffusion to adhesion complex dynamics

Mobile fractions of various IAC components were measured to assess their dynamics at the MTJs. In the case of integrins, the mobile fraction could be a measurement of turnover (assembly and disassembly) of the IAC or, alternatively, of lateral diffusion. Our FRAP experiments on whole and partial MTJs demonstrate that lateral mobility is not a significant factor contributing to the integrin dynamics we measured. 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 to and depart from the already assembled adhesion complex. For example, a recent study found that the FA plaque proteins paxillin and vinculin exist in four dynamic states: an immobile FA-bound fraction, an FA-associated fraction undergoing exchange, a juxtamembrane fraction experiencing 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 cytoplasmic components, we propose that the mobile fraction observed in the MTJ is mainly due to IAC assembly and disassembly, rather than diffusion and exchange. We suggest this based on two of our observations. First, we measured the fluorescence recovery of IAC components reaching their mobile fractions in 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 by free diffusion and occur on a similar timescale (Digman et al., 2008; Wolfenson et al., 2009). Second, 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 mobile fractions of both ILK and βPS-integrin significantly decline upon blockage of endocytosis. Nevertheless, it is still possible that ILK undergoes exchange; this might account for some of the 20% of the ILK protein that remained in the mobile fraction when clathrin-mediated endocytosis was inhibited.

Rab5 regulates integrin turnover

We have shown that Rab5 concentrates at MTJs and can regulate the size of the mobile fraction of IAC molecules that are undergoing turnover. This is consistent with published results showing that other Rab proteins, such as Rab21, regulate adhesion. In migrating cells, overexpression of Rab21 leads to increased integrin adhesion, whereas decreased expression of Rab21 leads to reduced adhesion (Pellinen and Ivaska, 2006; Tang and Ng, 2009). Intriguingly, MTJ defects are conferred by the expression of either Rab5-DN, which decreases the mobile fraction, or Rab5-CA, which increases the mobile fraction. It is not clear why the expression of either DN or CA versions of Rab5 gave rise to a nearly identical phenotype. However, our findings are consistent with previous work in flies showing that overexpression of integrins gives rise to muscle-detachment phenotypes identical to those found in integrin null mutants (Tanentzapf et al., 2006). By extension, a small reduction or a small increase in the amount of immobile ECM-ligand-bound integrin conferred by expression of Rab5-DN or Rab5-CA could lead to a similar muscle defect. 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 a careful balance between the process of integrin internalization and IAC disassembly, and the process of integrin trafficking to the MTJ and IAC assembly. Any deviation from the required balance between adhesion complex assembly and disassembly leads to muscle detachment.

Developmental regulation of IAC turnover at the MTJ

At the end of muscle morphogenesis (stage 16 of embryogenesis), IACs in muscles exhibit high rates of turnover similar to those observed in migrating cells. One possible explanation is that, because muscle morphogenesis involves dynamic processes, such as cell migration and tissue rearrangement, it requires extensive IAC turnover. The high levels of turnover observed at the immediate conclusion of muscle morphogenesis are therefore 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 to allow growth and remodeling to take place during larval development. Moreover, we would predict that the substantial levels of turnover observed in late embryonic and early larval stages are generally unsustainable in mature MTJs. Furthermore, we conjecture that a gradual reduction in the level 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 and long-lasting adhesion.

In addition to supporting stabilization of the adhesion junctions, we speculate that the reduction in the proportion of integrin and IAC components that undergo turnover plays 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. 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 remodeling of the MTJs, for example in response to increased mechanical stress. Integrin turnover in the adult might also contribute to the repair of MTJs in response to accrued mechanical damage. Because of the presence of an exoskeleton in the adults, it is not currently possible to analyze integrin turnover using FRAP, but our data show 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., 2010).

Models of IAC turnover

Based on our data, we propose that, in order to maintain the MTJs, the level of IAC turnover in the fully assembled muscle must be limited to within a specific range. This level of turnover necessitates equilibrium between IAC disassembly and IAC assembly. There are three generalized models for the turnover of the IAC: in one case, the entire complex is disassembled and assembled as a set unit; the second is that some of the IAC remains assembled and that only integrin molecules are internalized; the third is a mixture of both. Our experiments show that an increase or decrease in the mobile fraction of integrin was correlated with a similar increase or decrease in the turnover of other IAC components. Especially striking in this regard were the coordinated developmental changes in the mobile fractions of individual IAC components that occur during larval stages. This suggests that the turnover of multiple IAC components is co-regulated, which makes it unlikely that only integrins recycle while the rest of the complex remains intact.

Previous work has implicated focal adhesion kinase (FAK) and Src family kinases in regulating the dynamics of integrin-mediated adhesion (Ulrich and Heisenberg, 2009; Webb et al., 2002). However, expressing a dominant-negative version of Src in fly muscles induced early muscle defects, whereas disrupting FAK did not affect IAC turnover (our unpublished data). An important future goal is to identify the mechanism by which turnover is controlled in order to gain further insight into how IAC dynamics are modulated during development.

Conclusion

We hypothesize that modulating the levels of integrin turnover in the context of a stable long-term adhesive contact, such as the MTJ, provides a way for tissues to respond to changes in the external environment without wholesale disassembly and assembly of the adhesive contact. The ongoing existence of MTJs in a dynamic state enables expansion, contraction, remodeling and changes in the molecular components of the adhesion complex. This provides a flexibility that is vitally important for long-term tissue maintenance.

Materials and Methods

Molecular biology

The genomic rescue construct Ubi::talin-GFP was made using the Ubi::talin vector (Tanentzapf and Brown, 2006). The enhanced GFP (EGFP)-coding region was amplified from the pEGFP-N1 vector by PCR and *Mlu*I restriction enzyme sites were added flanking the coding sequence. EGFP was then inserted near the end of the talin-coding region after amino acid 2756. Ubi::βPS-YFP (integrin-YFP) was created from the pHSβPS-Venus plasmid (gift of Thomas Bunch, University of Arizona, Tucson, AZ), which contains a cDNA clone of βPS 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 4783 base pair section incorporating the *hsp70* promoter, the βPS-YFP fusion protein and the tubulin polyA site was excised from the plasmid using the restriction enzyme *Xba*I and inserted into the *Spe*I site downstream of the ubiquitin-63E promoter in the pWRpAUbiP plasmid (Tanentzapf and Brown, 2006). The *hsp70* promoter was then excised using *Sac*II and *Sgr*AI. Transgenics were generated by BestGene (Chino Hills, CA).

Fly stocks and lines

Stocks used in this study were: UAS-*shi*^{ts} (Kitamoto, 2001; BL5811), UAS-Rab5.S43N, UAS-Rab5, UAS-Rab5.Q88L, UAS-Rab2 and Mef2::Gal4 (Bloomington Drosophila Stock Center). Mef2::Gal4 was used to drive UAS expression in muscles. The *ilk*::GFP lines (ZCL3111, ZCL3192) were previously described by Hudson et al. (Hudson et al., 2008); the *blistery*::GFP line (tensin-GFP) is described in Torgler et al. (Torgler et al., 2004).

FRAP experiments and statistical analysis

Embryos used for FRAP analysis were collected from apple juice plates, dechorinated in 50% bleach for 5 minutes, and washed with PBT followed by PBS. Embryos were then mounted on glass slides in PBS. FRAP analysis was carried out at room temperature on whole-mount embryos or on whole-mount larvae 2 hours after mounting. Experiments using UAS-*shi*^{ts} and Rab GTPase constructs were carried out in flies heterozygous for integrin-YFP to allow the combination of multiple transgenes in one fly. Heterozygous integrin-YFP exhibits higher levels of turnover compared to homozygous integrin-YFP in flies at the same stage. All other analysis of integrin-YFP was carried out in homozygous integrin-YFP flies.

To study *shi*^{ts} flies, both the experimental group and the control group (without the UAS-*shi*^{ts} transgene) were heat shocked (30°C for embryos and 37°C for larvae) for an hour before FRAP analysis. FRAP was performed on a confocal microscope (FV1000; Olympus) with an UplanSApo 60×/1.35 oil objective (Olympus) and fully opened pinhole at 30°C or 37°C in an on-stage incubator. FRAP analysis of flat preparations was performed at room temperature immediately after mounting. Because 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 with the Tornado scanning tool (Olympus) for 2 seconds at 100 μs/pixel on all flat preparation experiments.

For all experiments other than the flat preparation, photobleaching was performed using the 405 nm laser at 30% power with the Tornado scanning tool (Olympus) for 2 seconds at 100 μs/pixel. Fluorescence recovery was recorded for 75 frames, with an interval of 4 seconds between each two frames. 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 8 μs/pixel.

To control muscle twitching in and out of focus, multiple regions of interest (ROI) were selected in non-photobleached regions; only samples for which intensities within control ROIs remained steady throughout the FRAP experiment were used. Recovery data were further analyzed using Prism (GraphPad, La Jolla, CA): mobile fraction and $t_{1/2}$ were calculated as previously described (Reits and Neefjes, 2001). Statistical tests (*t*-test, ANOVA test) were carried out using Prism.

Larval flat preparations

3rd instar larvae were dissected in modified HL3 medium (Kasprowicz et al., 2008). Dissected larva fillets were incubated in Schneider's medium with either 50 μM CPZ (Sigma-Aldrich) or 0.4 mM 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-bottomed 35 mm Petri dish. FRAP was then performed on the mounted fillets.

Analysis of MTJ width

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.

Immunohistochemistry and microscopy

Antibody staining was carried out according to standard procedures. Heat fixation (Tanentzapf and Brown, 2006) was used for all embryonic staining to enable staining of late-stage embryos. The following antibodies were used: anti-αPS2 [7A10 (Bogaert et al., 1987)], rat mAb (1:10); anti-talin [E16B (Brown et al., 2002)], mouse mAb (1:10); anti-tiggrin (Fogerty et al., 1994), mouse pAb (1:500) (gift of Liselotte Fessler, University of California, Los Angeles, CA); anti-paxillin, rabbit pAb (1:500); anti-MHC, mouse mAb (1:200) (gift of Daniel Kiehart, Duke University, Durham, NC); anti-GFP (A6455), rabbit pAb (1:1000) (Invitrogen). Images were collected using an Olympus FV1000 confocal microscope with an UplanSApo 60×/1.35 oil objective and processed using Adobe Photoshop.

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References

Balzac, F., Avolio, M., Degani, S., Kaverina, I., Torti, M., Silengo, L., Small, J. V. and Retta, S. F. (2005). E-cadherin endocytosis regulates the activity of Rap1: a traffic light GTPase at the crossroads between cadherin and integrin function. *J. Cell Sci.* **118**, 4765-4783.

Bate, M. (1993). The mesoderm and its derivatives. In *The Development of Drosophila melanogaster* (eds M. Bate and A. Martinez-Arias), pp. 1013-1090. New York: Cold Spring Harbor Laboratory Press.

Blitzer, J. T. and Nusse, R. (2006). A critical role for endocytosis in Wnt signaling. *BMC Cell Biol.* **7**, 28.

Bogaert, T., Brown, N. and Wilcox, M. (1987). The Drosophila PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. *Cell* **51**, 929-940.

Bretscher, M. S. (1989). Endocytosis and recycling of the fibronectin receptor in CHO cells. *EMBO J.* **8**, 1341-1348.

Bretscher, M. S. (1992). Circulating integrins: alpha 5 beta 1, alpha 6 beta 4 and Mac-1, but not alpha 3 beta 1, alpha 4 beta 1 or LFA-1. *EMBO J.* **11**, 405-410.

Brown, N. H. (1993). Integrins hold Drosophila together. *BioEssays* **15**, 383-390.

Brown, N. H., Gregory, S. L. and Martin-Bermudo, M. D. (2000). Integrins as mediators of morphogenesis in Drosophila. *Dev. Biol.* **223**, 1-16.

Brown, N. H., Gregory, S. L., Rickoll, W. L., Fessler, L. I., Prout, M., White, R. A. and Fristrom, J. W. (2002). Talin is essential for integrin function in Drosophila. *Dev. Cell* **3**, 569-579.

Caswell, P. T. and Norman, J. C. (2006). Integrin trafficking and the control of cell migration. *Traffic* **7**, 14-21.

Caswell, P. T., Spence, H. J., Parsons, M., White, D. P., Clark, K., Cheng, K. W., Mills, G. B., Humphries, M. J., Messent, A. J., Anderson, K. I. et al. (2007). Rab25 associates with alpha5beta1 integrin to promote invasive migration in 3D microenvironments. *Dev. Cell* **13**, 496-510.

Caswell, P. T., Chan, M., Lindsay, A. J., McCaffrey, M. W., Boettiger, D. and Norman, J. C. (2008). Rab-coupling protein coordinates recycling of alpha5beta1 integrin and EGFR1 to promote cell migration in 3D microenvironments. *J. Cell Biol.* **183**, 143-155.

Digman, M. A., Brown, C. M., Horwitz, A. R., Mantulin, W. W. and Gratton, E. (2008). Paxillin dynamics measured during adhesion assembly and disassembly by correlation spectroscopy. *Biophys. J.* **94**, 2819-2831.

Dinneen, J. L. and Ceresa, B. P. (2004). Continual expression of Rab5(Q79L) causes a ligand-independent EGFR internalization and diminishes EGFR activity. *Traffic* **5**, 606-615.

Duband, J. L., Nuckolls, G. H., Ishihara, A., Hasegawa, T., Yamada, K. M., Thiery, J. P. and Jacobson, K. (1988). Fibronectin receptor exhibits high lateral mobility in embryonic locomoting cells but is immobile in focal contacts and fibrillar streaks in stationary cells. *J. Cell Biol.* **107**, 1385-1396.

Edlund, M., Lotano, M. A. and Otey, C. A. (2001). Dynamics of alpha-actinin in focal adhesions and stress fibers visualized with alpha-actinin-green fluorescent protein. *Cell Motil. Cytoskeleton* **48**, 190-200.

Ezratty, E. J., Partridge, M. A. and Gundersen, G. G. (2005). Microtubule-induced disassembly is mediated by dynamin and kinase. *Nat. Cell Biol.* **7**, 581-590.

Fogerty, F. J., Fessler, L. I., Bunch, T. A., Yaron, Y., Parker, C. G., Nelson, R. E., Brower, D. L., Gullberg, D. and Fessler, J. H. (1994). Tiggrin, a novel Drosophila extracellular matrix protein that functions as a ligand for Drosophila alpha PS2 beta PS integrins. *Development* **120**, 1747-1758.

Hayashi, Y. K., Chou, F. L., Engvall, E., Ogawa, M., Matsuda, C., Hirabayashi, S., Yokochi, K., Ziober, B. L., Kramer, R. H., Kaufman, S. J. et al. (1998). Mutations in the integrin alpha7 gene cause congenital myopathy. *Nat. Genet.* **19**, 94-97.

Hudson, A. M., Petrella, L. N., Tanaka, A. J. and Cooley, L. (2008). Mononuclear muscle cells in Drosophila ovaries revealed by GFP protein traps. *Dev. Biol.* **314**, 329-340.

Kaisto, T., Rahkila, P., Marjomaki, V., Parton, R. G. and Metsikko, K. (1999). Endocytosis in skeletal muscle fibers. *Exp. Cell Res.* **253**, 551-560.

Kasprowicz, J., Kuenen, S., Miskiewicz, K., Habets, R. L., Smitz, L. and Verstreken, P. (2008). Inactivation of clathrin heavy chain inhibits synaptic recycling but allows bulk membrane uptake. *J. Cell Biol.* **182**, 1007-1016.

Kitamoto, T. (2001). Conditional modification of behavior in Drosophila by targeted expression of a temperature-sensitive shibire allele in defined neurons. *J. Neurobiol.* **47**, 81-92.

Liu, S., Calderwood, D. A. and Ginsberg, M. H. (2000). Integrin cytoplasmic domain-binding proteins. *J. Cell Sci.* **113**, 3563-3571.

Macia, E., Ehrlich, M., Massol, R., Boucrot, E., Brunner, C. and Kirchhausen, T. (2006). Dynasore, a cell-permeable inhibitor of dynamin. *Dev. Cell* **10**, 839-850.

Mayer, U., Saher, G., Fassler, R., Bornemann, A., Echtermeyer, F., von der Mark, H., Miosge, N., Poschl, E. and von der Mark, K. (1997). Absence of integrin alpha 7 causes a novel form of muscular dystrophy. *Nat. Genet.* **17**, 318-323.

McKenna, N. M., Meigs, J. B. and Wang, Y. L. (1985). Exchangeability of alpha-actinin in living cardiac fibroblasts and muscle cells. *J. Cell Biol.* **101**, 2223-2232.

Morin, X., Daneman, R., Zavortink, M. and Chia, W. (2001). A protein trap strategy to detect GFP-tagged proteins expressed from their endogenous loci in Drosophila. *Proc. Natl. Acad. Sci. USA* **98**, 15050-15055.

Nielsen, E., Severin, F., Backer, J. M., Hyman, A. A. and Zerial, M. (1999). Rab5 regulates motility of early endosomes on microtubules. *Nat. Cell Biol.* **1**, 376-382.

Pellinen, T. and Ivaska, J. (2006). Integrin traffic. *J. Cell Sci* **119**, 3723-3731.

Pellinen, T., Arjonen, A., Vuoriluoto, K., Kallio, K., Fransen, J. A. and Ivaska, J. (2006). Small GTPase Rab21 regulates cell adhesion and controls endosomal traffic of beta1-integrins. *J. Cell Biol.* **173**, 767-780.

Perkins, A. D., Ellis, S. J., Asghari, P., Shamsian, A., Moore, E. D. and Tanentzapf, G. (2010). Integrin-mediated adhesion maintains sarcomeric integrity. *Dev. Biol*. **338**, 15-27.

Reits, E. A. and Neefjes, J. J. (2001). From fixed to FRAP: measuring protein mobility and activity in living cells. *Nat. Cell Biol.* **3**, E145-E147.

Roberts, M., Barry, S., Woods, A., van der Sluijs, P. and Norman, J. (2001). PDGF-regulated rab4-dependent recycling of alphavbeta3 integrin from early endosomes is necessary for cell adhesion and spreading. *Curr. Biol.* **11**, 1392-1402.

Tanentzapf, G. and Brown, N. H. (2006). An interaction between integrin and the talin FERM domain mediates integrin activation but not linkage to the cytoskeleton. *Nat. Cell Biol.* **8**, 601-606.

Tanentzapf, G., Martin-Bermudo, M. D., Hicks, M. S. and Brown, N. H. (2006). Multiple factors contribute to integrin-talin interactions in vivo. *J. Cell Sci.* **119**, 1632-1644.

Tang, B. L. and Ng, E. L. (2009). Rabs and cancer cell motility. *Cell Motil. Cytoskeleton*. **66**, 365-370.

Torgler, C. N., Narasimha, M., Knox, A. L., Zervas, C. G., Vernon, M. C. and Brown, N. H. (2004). Tensin stabilizes integrin adhesive contacts in Drosophila. *Dev. Cell* **6**, 357-369.

Trushina, E., Singh, R. D., Dyer, R. B., Cao, S., Shah, V. H., Parton, R. G., Pagano, R. E. and McMurray, C. T. (2006). Mutant huntingtin inhibits clathrin-independent endocytosis and causes accumulation of cholesterol in vitro and in vivo. *Hum. Mol. Genet.* **15**, 3578-3591.

Tsuruta, D., Gonzales, M., Hopkinson, S. B., Otey, C., Khuon, S., Goldman, R. D. and Jones, J. C. (2002). Microfilament-dependent movement of the beta3 integrin subunit within focal contacts of endothelial cells. *FASEB J.* **16**, 866-868.

Ulrich, F. and Heisenberg, C. P. (2009). Trafficking and cell migration. *Traffic* **10**, 811-818.

Wang, L. H., Rothberg, K. G. and Anderson, R. G. (1993). Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *J. Cell Biol.* **123**, 1107-1117.

Webb, D. J., Parsons, J. T. and Horwitz, A. F. (2002). Adhesion assembly, disassembly and turnover in migrating cells - over and over and over again. *Nat. Cell Biol.* **4**, E97-E100.

Webb, D. J., Brown, C. M. and Horwitz, A. F. (2003). Illuminating adhesion complexes in migrating cells: moving towards a bright future. *Curr. Opin. Cell Biol.* **15**, 614-620.

White, D. P., Caswell, P. T. and Norman, J. C. (2007). alphavbeta3 and alpha5beta1 integrin recycling pathways dicatate downstream Rho kinases signaling to regulate persistent cell migration. *J. Cell Biol.* **177**, 515-525.

Wolfenson, H., Lubelski, A., Regev, T., Klafter, J., Henis, Y. I. and Geiger, B. (2009). A role for the juxtamembrane cytoplasm in the molecular dynamics of focal adhesions. *PLoS ONE* **4**, e4304.

Woods, A. J., White, D. P., Caswell, P. T. and Norman, J. C. (2004). PKD1/PKCmu promotes alphavbeta3 Integrin recycling and delivery to nascent focal adhesions. *EMBO J.* **23**, 2531-2543.

Zerial, M. and McBride, H. (2001). Rab proteins as membrane organizers. *Nat. Rev. Mol. Cell. Biol.* **2**, 107-117.

Zhang, J., Schulze, K. L., Hiesinger, P. R., Suyama, K., Wang, S., Fish, M., Acar, M., Hoskins, R. A., Bellen, H. J. and Scott, M. P. (2007). Thirty-one flavors of Drosophila rab proteins. *Genetics* **176**, 1307-1322.