

RESEARCH ARTICLE

Direct binding of Talin to Rap1 is required for cell–ECM adhesion in *Drosophila*

Darius Camp¹, Amanda Haage¹, Veronika Solianova¹, William M. Castle², Qinyuan A. Xu¹, Emily Lostchuck¹, Benjamin T. Goult² and Guy Tanentzapf^{1,*}

ABSTRACT

Attachment of cells to the extracellular matrix (ECM) via integrins is essential for animal development and tissue maintenance. The cytoplasmic protein Talin (encoded by *rhea* in flies) is necessary for linking integrins to the cytoskeleton, and its recruitment is a key step in the assembly of the adhesion complex. However, the mechanisms that regulate Talin recruitment to sites of adhesion *in vivo* are still not well understood. Here, we show that Talin recruitment to, and maintenance at, sites of integrin-mediated adhesion requires a direct interaction between Talin and the GTPase Rap1. A mutation that blocks the direct binding of Talin to Rap1 abolished Talin recruitment to sites of adhesion and the resulting phenotype phenocopies that seen with null alleles of Talin. Moreover, we show that Rap1 activity modulates Talin recruitment to sites of adhesion via its direct binding to Talin. These results identify the direct Talin–Rap1 interaction as a key *in vivo* mechanism for controlling integrin-mediated cell–ECM adhesion.

KEY WORDS: Cell adhesion, *Drosophila*, Morphogenesis, ECM, Integrin, Talin, Rap1

INTRODUCTION

The assembly and maintenance of tissue architecture is dependent on large multiprotein cell adhesion complexes. This presents a challenge to cells, as they need to coordinate the delivery of a large number of diverse proteins to specific sites at the cell membrane. Importantly, the process of recruiting components of the complex to nascent sites of adhesion and assembling them into a functional unit provides a valuable opportunity for regulating cell adhesion. For these reasons, there has been a great deal of interest in understanding the mechanisms that control recruitment and delivery of adhesion complex components to the cell cortex. In the case of integrin-mediated cell–extracellular matrix (ECM) adhesion, protein recruitment to the cell cortex is regulated by mechanisms that involve trafficking machinery and modulation of protein–protein interactions within the complex (Paul et al., 2015; Wehrle-Haller, 2012). In recent years, evidence has accumulated from *in vivo* studies that the regulation of integrin adhesion complex dynamics is important during tissue development and maintenance (Costa and Parsons, 2010; Daley and Yamada, 2013; Wolfenson et al., 2013). Consequently, the use of mutations that alter

the strength and stability of integrin adhesions provide a valuable opportunity to interrogate the role of cell–ECM attachment for a wide array of developmental processes.

The protein Talin (encoded by *rhea* in flies; *Tln1* and *Tln2* in mammals) is a central component of the integrin adhesion complex and is essential for the assembly and maintenance of integrin-based cell–ECM attachment (Klapholz and Brown, 2017). Talin binds to the β -integrin cytoplasmic tail directly and then links integrins to the cytoskeleton either directly, through its actin-binding domains (Franco-Cea et al., 2010) or indirectly, by recruiting downstream components of the adhesion complex (Giannone et al., 2003; Tanentzapf et al., 2006). In addition, Talin plays an important role in regulating the affinity of integrins for their ECM ligands through regulating integrin activation (Shattil et al., 2010; Tadokoro et al., 2003). Talin contains two known integrin-binding sites (IBSs): IBS-1 is located in the N-terminal end of the protein, while IBS-2 is in the C-terminus. The IBS-1 domain of Talin, also known as the Talin head domain, has been extensively implicated in integrin activation. Expression of IBS-1 is sufficient to activate integrins in diverse contexts (Calderwood et al., 1999; Ellis et al., 2011). It is thought that the binding of the Talin IBS-1 to the β -integrin cytoplasmic tail causes a change in the angle of the tail relative to the plasma membrane, disrupting interactions between the tails of the α - and β -integrin, and inducing integrin activation (Wegener et al., 2007). The mechanisms that control the recruitment of Talin to sites of adhesion at the cell cortex are therefore important for the regulation of integrin function (Calderwood et al., 2013; Klapholz and Brown, 2017).

Another important, and well characterized, protein that regulates integrin function is the small GTPase Rap1 (Rap1a and Rap1b in mammals). Rap1 is known to be an activator of integrin function in diverse biological contexts (Boettner and Van Aelst, 2009). Currently, Rap1-mediated integrin activation is thought to occur via a complex made up of Rap1, the Rap1 effector Rap1-GTP-interacting adaptor molecule (RIAM; also known as APBB1IP in mammals and Pico in flies) and Talin. RIAM is an adaptor molecule whose main function has been proposed to be the targeting of Talin to integrins (Han et al., 2006). Rap1 binds directly to RIAM through its Ras-association domain, and RIAM binds to Talin through 30 residues at its N-terminal. The complex is recruited to the cell cortex through a membrane-targeting sequence in Rap1. Intriguingly, a construct containing only a fusion of the membrane-targeting sequence of Rap1 and the Talin-binding sequence of RIAM is fully sufficient to recruit Talin to the membrane and induce integrin activation (Lee et al., 2009). Nonetheless, recent evidence has suggested that in many contexts RIAM is dispensable for Talin recruitment to sites of adhesion (Stritt et al., 2015). This raises the possibility that Rap1 might regulate integrin activity through Talin via an alternative mechanism. More recently, evidence has emerged that Rap1 can bind Talin directly and regulate its recruitment to the membrane. Biochemical experiments have already shown weak

¹Department of Cellular and Physiological Sciences, University of British Columbia, Vancouver, Canada V6T 1Z3. ²School of Biosciences, University of Kent, Canterbury CT2 7NJ, UK.

*Author for correspondence (tanentz@mail.ubc.ca)

© D.C., 0000-0003-1948-6330; A.H., 0000-0001-6305-440X; B.T.G., 0000-0002-3438-2807; G.T., 0000-0002-2443-233X

direct binding between Talin and Rap1, and suggested this interaction can serve as a mechanism for targeting Talin to the membrane (Goult et al., 2010; Zhu et al., 2017). Furthermore, in the slime mould *Dictyostelium*, direct binding of Rap1 to Talin was shown to be necessary for adhesion during multicellular development (Plak et al., 2016). However, whether the direct binding of Rap1 to Talin is relevant in complex multicellular organisms, and if so in what contexts, has not been established.

Drosophila serves as a powerful model system for studying the function of components of the integrin adhesion complex. In particular, the fly has proven to be useful for *in vivo* structure–function analysis of components of the integrin adhesion complex. Loss-of-function mutations in *mysospheroid* (*mys*), the gene that encodes the main β -integrin subunit in flies, cause severe embryonic defects in multiple tissues (Leptin et al., 1989). Among the best-characterized integrin-dependent processes in the fly is the stable attachment of muscle cells to tendon cells through the ECM in prominent integrin-based adhesions at myotendinous junctions (MTJs) (Schweitzer et al., 2010). Additionally, two dynamic integrin-adhesion based morphogenetic processes, germband retraction (GBR) and the wound closure-like process of dorsal closure (DC), have been analysed in detail (Narasimha and Brown, 2004; Schöck and Perrimon, 2003). Forward genetics screens in the fly have led to the isolation and characterization of mutations in more than a dozen genes that encode cytoplasmic factors involved in integrin function, including, PINCH (Clark et al., 2003), Paxillin (Yagi et al., 2001), Tensin (Torgler et al., 2004), ILK (Zervas et al., 2001) and Talin (Brown et al., 2002). *Drosophila* Talin is particularly important for integrin-mediated adhesion in flies. Flies lacking Talin exhibit a phenotype that is largely indistinguishable from that observed following loss of integrins. In Talin mutants, muscle attachment to tendon cells fails, and both GBR and DC are disrupted (Brown et al., 2002). The fly has proven to be a particularly useful system in which to analyse Talin function, and detailed structure–function analysis of specific domains of Talin has provided mechanistic insight into its regulation during tissue development and homeostasis (Ellis et al., 2011, 2013; Franco-Cea et al., 2010; Tanentzapf and Brown, 2006).

Here, we have characterized the role of direct binding of Talin to Rap1 *in vivo*. Through a CRISPR/Cas9-based strategy, we introduced a point mutation that specifically blocks the direct binding of Talin to Rap1 into genomic Talin in *Drosophila*. We find that disrupting the ability of Talin to directly bind Rap1 completely abolished its function. In these mutants, Talin was not recruited to sites of adhesion, and comprehensive phenotypic analysis confirmed that the resulting phenotype was very similar to that seen in Talin-null flies. Furthermore, by using Talin and Rap1 transgenes, we provide evidence showing that Rap1 activity regulates Talin recruitment to the membrane via their direct interaction. This work establishes that direct binding of Talin and Rap1 regulates Talin recruitment to sites of adhesion, and is thus an essential regulator of adhesion complex assembly and maintenance *in vivo*.

RESULTS

Direct binding of the Talin F0 domain to Rap1 is conserved in flies

Several studies have defined a region in Talin that binds directly to Rap1 (Goult et al., 2010; Plak et al., 2016; Zhu et al., 2017). The Talin head domain is composed of the four subdomains F0, F1, F2 and F3, with F1–F3 making up the FERM (4.1/ezrin/radixin/moesin)-like domain (Fig. 1A). The site of interaction between Rap1 and Talin lies in the F0 domain. This region is required for integrin activation

(Bouaouina et al., 2008) and is well conserved between fly and vertebrate Talin (Fig. 1B). Homology modelling showed that the binding interfaces of fly Rap1 and the Talin F0 domain are very similar to those shown to be important in the recently solved structure of vertebrate talin 1 bound to Rap1b (PDB ID 6ba6; Zhu et al., 2017). Notably, positively charged surface residues found in Rap1-binding domains are conserved in fly Talin (Fig. 1B–D; Goult et al., 2010). Nuclear magnetic resonance (NMR) experiments using ^{15}N -labelled fly Talin F0 and Rap1b revealed clear chemical shift changes when Rap1b was added to Talin F0, indicating a direct interaction (Fig. 1E). To further analyse this chemical shift data, we used a ^{13}C - ^{15}N -labelled F0 domain (residues 1–87) to complete the backbone assignment of Talin F0. The weighted ^1H , ^{15}N chemical shifts in F0 induced by Rap1b (Fig. 1E) were plotted as a function of residue number (Fig. S1) and plotted onto the structure of the F0–Rap1b complex (Fig. 1C,D). From this NMR and structural data, it was shown that a conserved lysine residue (K15 in vertebrate talin 1; K17 in fly Talin) was crucial for binding of Rap1 to the Talin F0 domain (Fig. 1B; Goult et al., 2010; Zhu et al., 2017). We introduced the equivalent K17E mutation into the fly Talin F0 and found that it did not affect the folding of the protein (Fig. S1). Importantly, NMR experiments confirmed that the K17E mutation in fly Talin completely abrogated F0 binding to Rap1 (Fig. 1F; Fig. S2). These results suggest that the direct binding of Talin to Rap1 through the Talin F0 domain is evolutionarily conserved and identifies K17E as an effective tool to disrupt this binding.

Generation of a Talin mutant that blocks the direct binding of the Talin F0 domain to Rap1

Next, we introduced the K17E mutation into *rhea*, the gene that encodes Talin in flies. We utilized CRISPR/Cas9-mediated homology-directed repair (HDR) with a double-stranded DNA (dsDNA) donor template to genetically engineer the endogenous *rhea* locus (Fig. 2). We employed two guide RNAs (gRNAs) targeting sites 5' and 3' of the sequence corresponding to K17, as well as a dsDNA donor with homology arms containing ~1 kb of sequence flanking the targeted cleavages sites in addition to the targeted point mutation (Fig. 2A). To select for targeted events, our donor vector also contained a visible DsRed eye marker flanked by loxP sites for CRE-mediated removal. The residual 34 bp loxP site leftover from the CRE recombination was specifically introduced in an area with a low degree of sequence conservation. Furthermore, previous studies have shown that even large 7.5 kb Minos insertions (Mi{ET}MB11781) had no noticeable effects on Talin function at this site (Fig. 2A; Bellen et al., 2004). Using this approach, we isolated a number of mutant lines. These were sequenced extensively both upstream and downstream of the K17E mutation site to confirm the presence of the mutation and to ensure no other deleterious events took place at the *rhea* locus (Fig. 2B,C). We noted that all the mutant *rhea*^{K17E} lines were embryonic lethal. Importantly, the *rhea*^{K17E} allele failed to complement null alleles of *rhea*. Moreover, introducing a ubiquitously expressing Talin rescue construct (ubi::Talin; Tanentzapf and Brown, 2006) into the background of null alleles of *rhea* or the *rhea*^{K17E} allele rescued the embryonic lethality associated with loss of Talin to the same extent (i.e. both flies were viable until pupation) (Fig. 2D). Taken together, these data show that the *rhea*^{K17E} allele behaves like a genetic null allele of Talin.

Blocking the direct binding of Rap1 to Talin through the F0 domain disrupts embryonic tissue morphogenesis

To further understand the nature of the phenotypes caused by introducing the K17E mutation in Talin, the embryonic phenotype

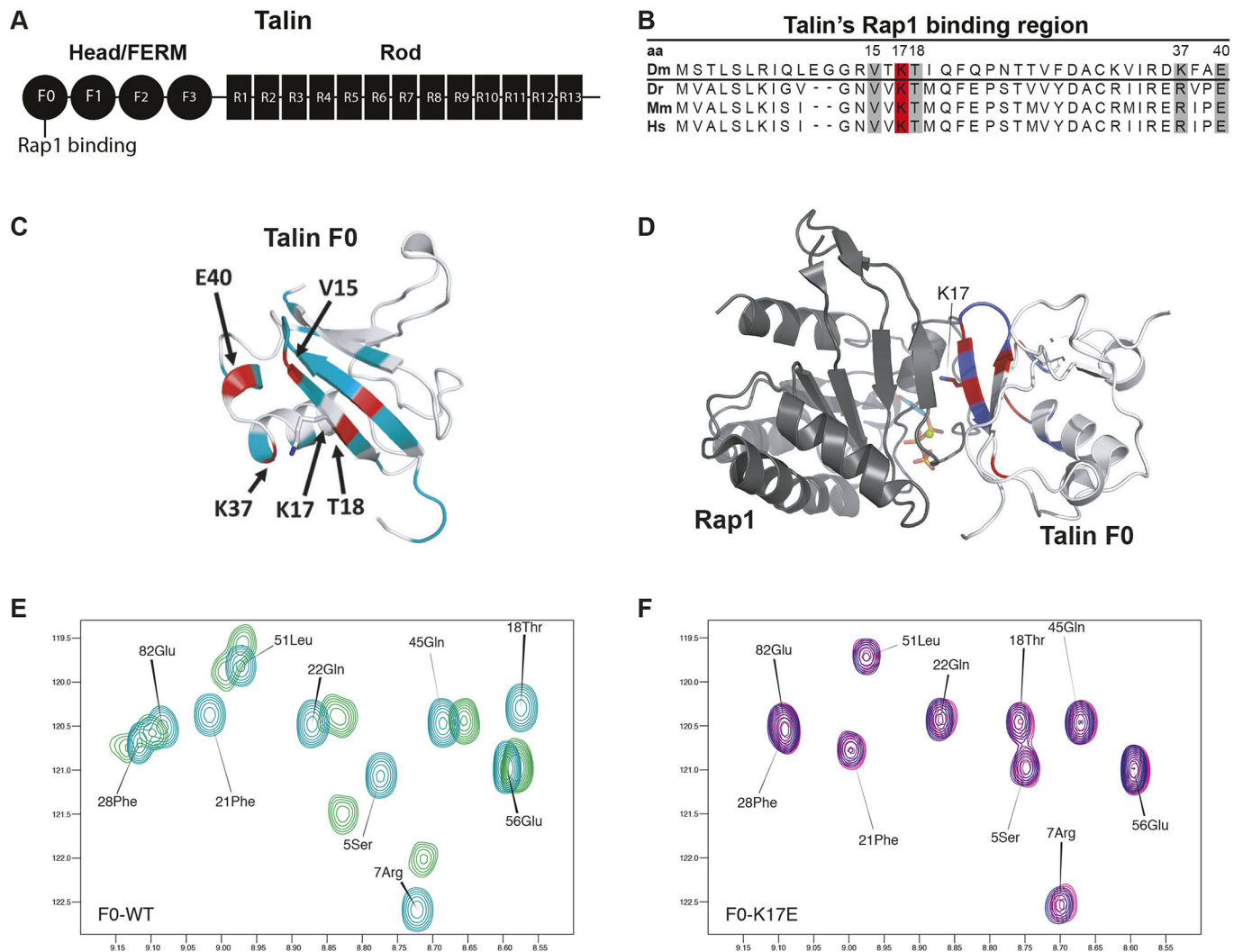


Fig. 1. Biochemical and structural characterization of the Rhea-F0 Rap1 interaction. (A) Schematic representation of the FERM and rod domains of Talin and the location of the Rap1-binding region in the F0 domain of the Talin head. (B) Sequence alignment of the Rap1 binding region from fly (Dm Talin), zebrafish (Dr Talin1), mouse (Mm talin 1) and human (Hs talin 1) talins. Amino acid numbering is based on Dm Talin. Shading (grey and red) indicates positively charged residues important for Rap1 binding. Red shading indicates the position of K17, the residue mutated in this study. (C) Mapping of the Rap1 binding site on Talin F0. Conserved residues shown in B are marked. (D) Structural model of the Talin-F0-Rap1 interaction. Weighted chemical shift differences, determined as described previously (Goult et al., 2009), are shown on ribbon representations of the F0-Rap1b structure; peaks that broaden are shown in blue, shifts of >0.13 ppm are in red. (E,F) ^1H , ^{15}N HSQC spectra of 50 μM ^{15}N -labelled Talin F0 (residues 1–87) in the absence or presence of Rap1b. (E) wild-type F0 alone (teal) and with Rap1 (green); (F) K17E F0 alone (pink) and with Rap1 (purple).

of the *rhea*^{K17E} allele was analysed in detail. Although loss of Talin impacts on diverse embryonic phenotypes, we focused on four integrin-dependent processes that provide a broad overview of integrin-mediated cell-ECM adhesion during development. Specifically, two dynamic morphogenetic processes, DC and GBR were analysed. In addition, two stable long-term adhesive processes, muscle attachment via the ECM to the epidermis at MTJs, and epithelial adhesion in the fly wing, were also studied.

Large integrin-based adhesions form at MTJs starting mid-way through fly embryogenesis (Fig. 3A,D). Loss of integrins or Talin results in loss of muscle attachment to the tendons at the MTJs and subsequent rounding of the muscles (Leptin et al., 1989; Fig. 3B,E,L). Severe disruption to MTJs, similar to that seen in a null allele of *rhea*, was observed in mutants for the *rhea*^{K17E} allele (Fig. 3C,F,L). Another similarity between mutants from the null and *rhea*^{K17E} alleles was that the full extent of the mutant phenotypes was observed only once the maternal contribution of wild-type Talin was

eliminated via the use of germline clones (see Materials and Methods), as the maternal contribution provided a substantial amount of phenotypic rescue (Fig. 3G–I,L). The morphogenetic movements of GBR, a coordinated cell movement involving rapid posterior and ventral movement of the germband, and DC, the closing of a large dorsal hole in the embryo, are both dependent on integrins and Talin (Brown et al., 2002; Narasimha and Brown, 2004; Schöck and Perrimon, 2003). Introducing the K17E mutation strongly disrupted DC and a comparable proportion of the embryos of either the null or the *rhea*^{K17E} genotype failed to complete the process (Fig. 3A–C,K,M). Similar results were obtained for GBR, as a nearly identical proportion of the embryos of either the null or *rhea*^{K17E} genotype failed to complete the process (Fig. 3A–C,J,N). As observed for the MTJ phenotype, both DC and GBR were fully rescued in the null and *rhea*^{K17E} alleles by the presence of the maternal wild-type Talin (Fig. 3M,N). Finally, the ability of integrin-mediated adhesion to support the attachment of the two

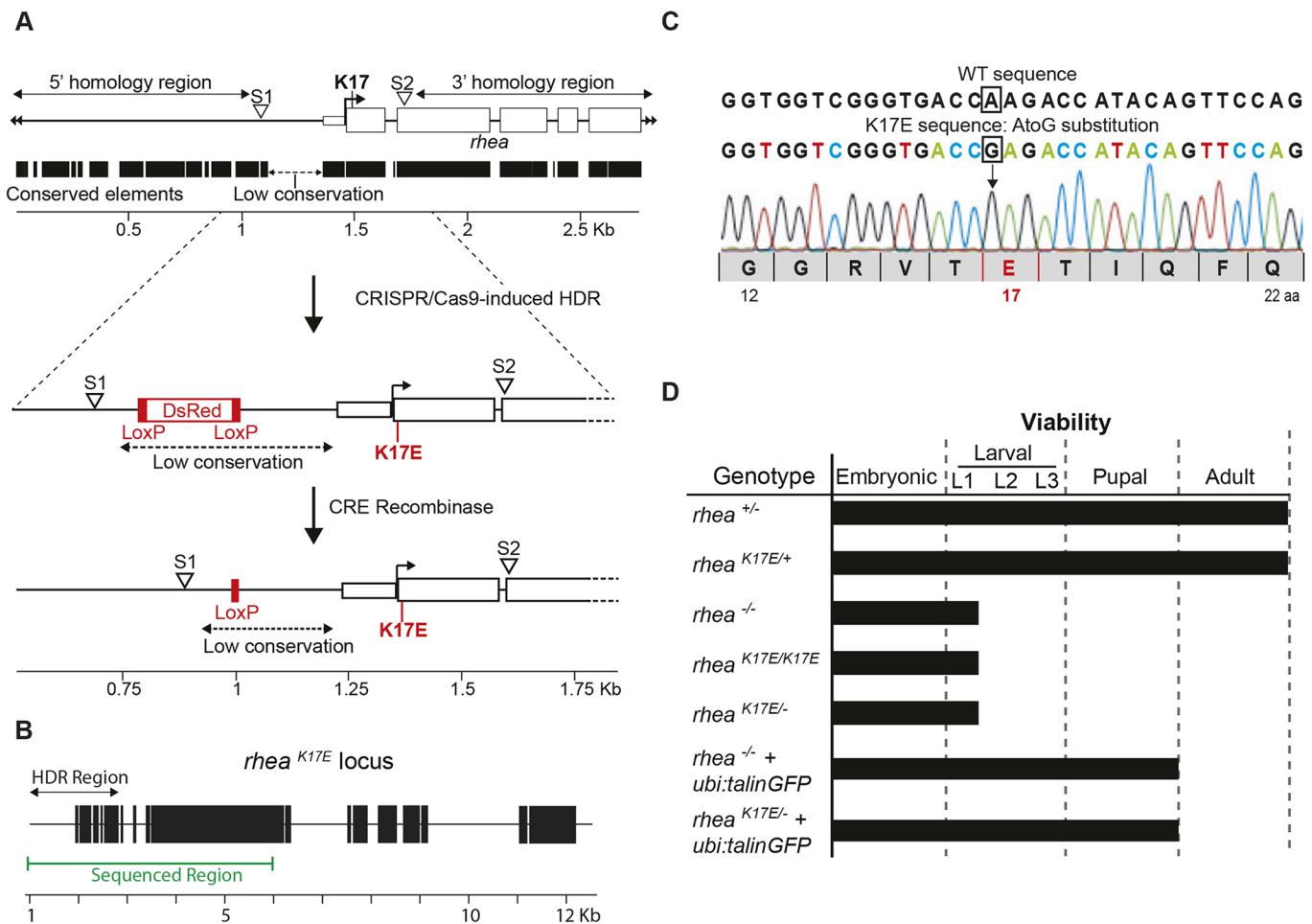


Fig. 2. Generation of the K17E Talin mutant. CRISPR strategy to introduce the K17E mutation into the *rhea* locus. S1 and S2 represent the cut sites targeted by Cas9, red elements represent inserted modifications (see Materials and Methods). (B) Schematic of the *rhea* locus; the sequenced region is shown in green. (C) Representative electropherogram of K17E mutant flies sequencing. (D) Viability of flies carrying the K17E mutation in genetic backgrounds as shown ($n > 100$ per genotype).

layers of epithelial cells that make up the fly wing was assayed by quantifying the proportion of flies with the characteristic wing-blistering defect that is caused by loss of cell–ECM adhesion. In this context, introducing the K17E mutation into Talin resulted in wing blistering in a similar proportion of flies to that seen with a null allele of Talin (Fig. 3O). Taken together, these data show that, at least in the context of tissue level phenotypes, introducing the K17E mutation into Talin abolishes its function to a similar extent to that seen with complete loss-of-function mutations in Talin.

Blocking the direct binding of Rap1 to Talin through the F0 domain disrupts the assembly of the integrin adhesion complex

The strong functional defects caused by the K17E mutation during fly development suggested a severe disruption to integrin-mediated adhesion. Such a defect could be caused by problems with the assembly of the adhesions. To analyse the adhesion complex assembly in detail, we employed a method to study colocalization of integrins with markers for the integrin adhesion complex (Ellis et al., 2011, 2013, 2014). Specifically, the distribution of integrins (Leptin et al., 1989), Paxillin (Yagi et al., 2001) and PINCH (Clark et al., 2003) was used to visualize the integrin adhesion complex in wild-type and mutant embryos (Fig. 4). In wild-type MTJs, both Paxillin and PINCH concentrated at the MTJs and their distribution

largely overlapped with that of integrins (Fig. 4A,G and D,J, respectively). In contrast, in embryos lacking Talin, integrins still localized to MTJs though at much lower levels than the wild-type, but both Paxillin and PINCH failed to concentrate at the MTJs (Fig. 4B,H and E,K, respectively). Similarly, mutant *rhea*^{K17E} embryos localized integrins weakly to MTJs, but both Paxillin and PINCH failed to concentrate at the MTJs (Fig. 4C,I and F,L, respectively). These results suggest Talin containing the K17E mutation was unable to support the assembly of the integrin adhesion complex.

Blocking the direct binding of Rap1 to Talin through the F0 domain affects Talin localization to the cell cortex

The strong loss-of-function phenotypes and the failure to assemble the integrin adhesion complex upon K17E mutation could result from changes in the production or localization of Talin. Analysis using quantitative PCR (qPCR) showed no statistically significant differences in levels of transcription of Talin RNA from either the wild-type or the engineered *rhea*^{K17E} locus (Fig. 5A). Furthermore, western blots showed no statistically significant differences in the overall levels of Talin protein in the wild-type or the *rhea*^{K17E} embryos (Fig. 5B,C). To analyse the relative stability of Talin with the K17E mutation at sites of adhesion in comparison to the wild-type version of the protein, transgenes were used that

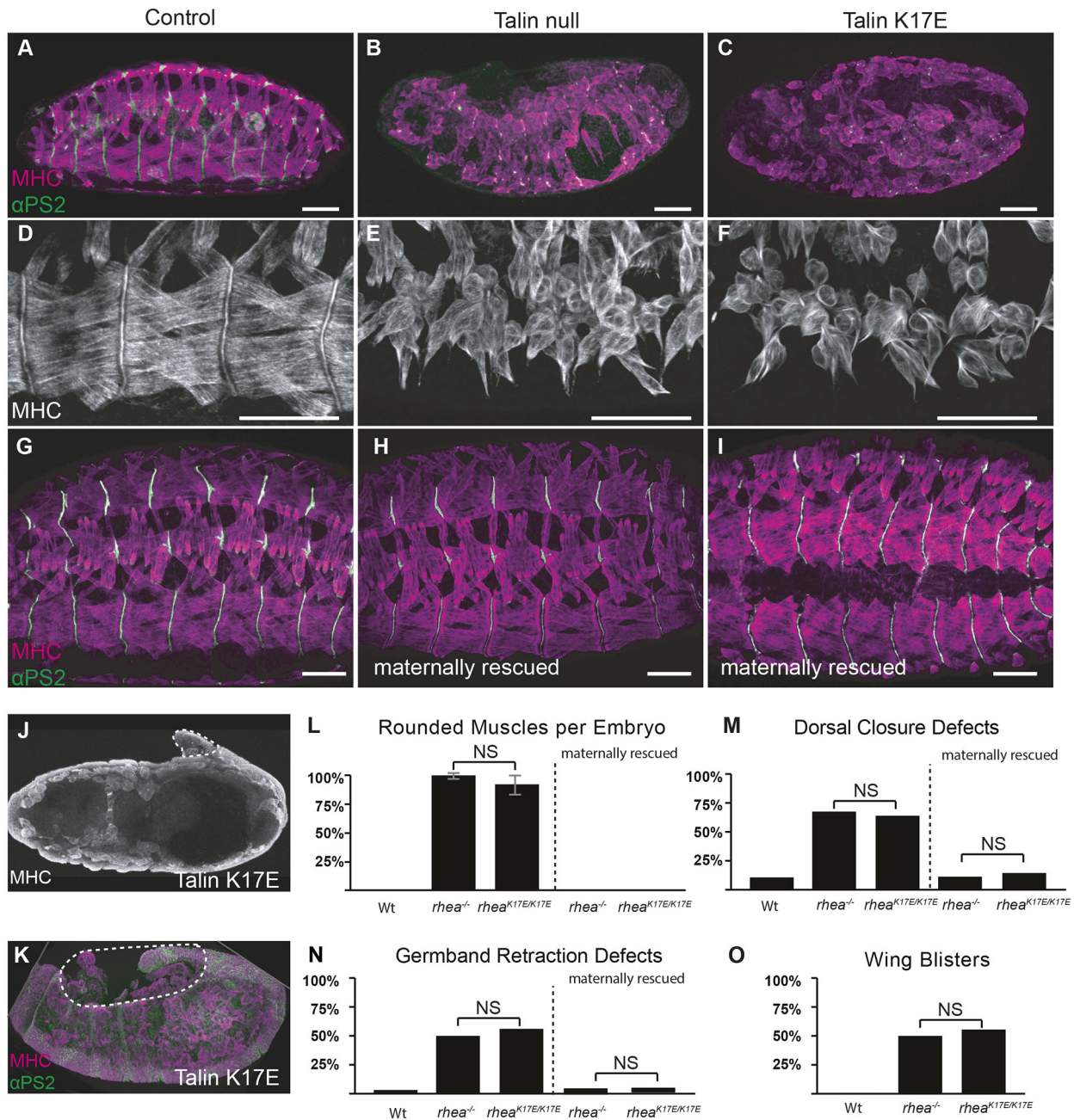


Fig. 3. Rap1 binding is strictly required for Talin function. (A–K) Confocal images of whole-mount embryos at stage 17. (A–C, G–I) Whole embryo view stained for the integrin subunit α PS2 (also known as Inflated) in green and the muscle cytoskeleton marker Myosin Heavy Chain (MHC) in magenta. (D–F) Representative muscles stained with MHC in hemisegments A2–A6. Control (A, D), Talin-null (B, E) and K17E embryos (C, F). Control embryo (G), Talin-null (H) and K17E (I) embryos with maternal contribution (see Materials and Methods). (J) Representative GBR defect giving the embryo a characteristic ‘tail up’ phenotype highlighted by the dotted line in a K17E embryo stained for MHC. (K) Representative DC defect (dotted line highlights persistent dorsal hole) in a K17E embryo stained for α PS2 (green) and MHC (magenta). (L–O) Penetrance of muscle (L), DC (M), GBR (N) and wing (O) defects in embryos of the indicated genotypes. Error bars represent s.e.m. NS, not significant ($n > 30$ for each genotype). Scale bars: 50 μ m.

contained a ubiquitously expressed full-length Talin construct that was either wild-type (ubi-talinGFP-WT; Tanentzapf and Brown, 2006) or contained the K17E mutation (ubi-talinGFP*K17E). Fluorescence recovery after photobleaching (FRAP) experiments were carried out with ubi-talinGFP-WT and ubi-talinGFP*K17E, in a background that also contained the wild-type untagged genomic Talin. These FRAP experiments showed a substantially higher mobile fraction for Talin containing the K17E mutation, consistent with reduced stability of the K17E mutant Talin at sites of adhesion (Fig. 5K).

Consistent with this result, there was a dramatic reduction of Talin at MTJs in *rhea*^{K17E} embryos compared to that seen in wild type (Fig. 5D–J). In wild-type MTJs, integrins and Talin colocalize, and appear as a discrete line at muscle attachments (Fig. 5G, J). In mutant embryos of either the null and *rhea*^{K17E} alleles, integrins were present at MTJs, although at notably lower levels compared to in wild type, and Talin was not detected in the MTJs (Fig. 5H–J). To account for the possibility that the defects in MTJ architecture in null or *rhea*^{K17E} mutant embryos underlie this recruitment phenotype, we analysed Talin recruitment in the background of

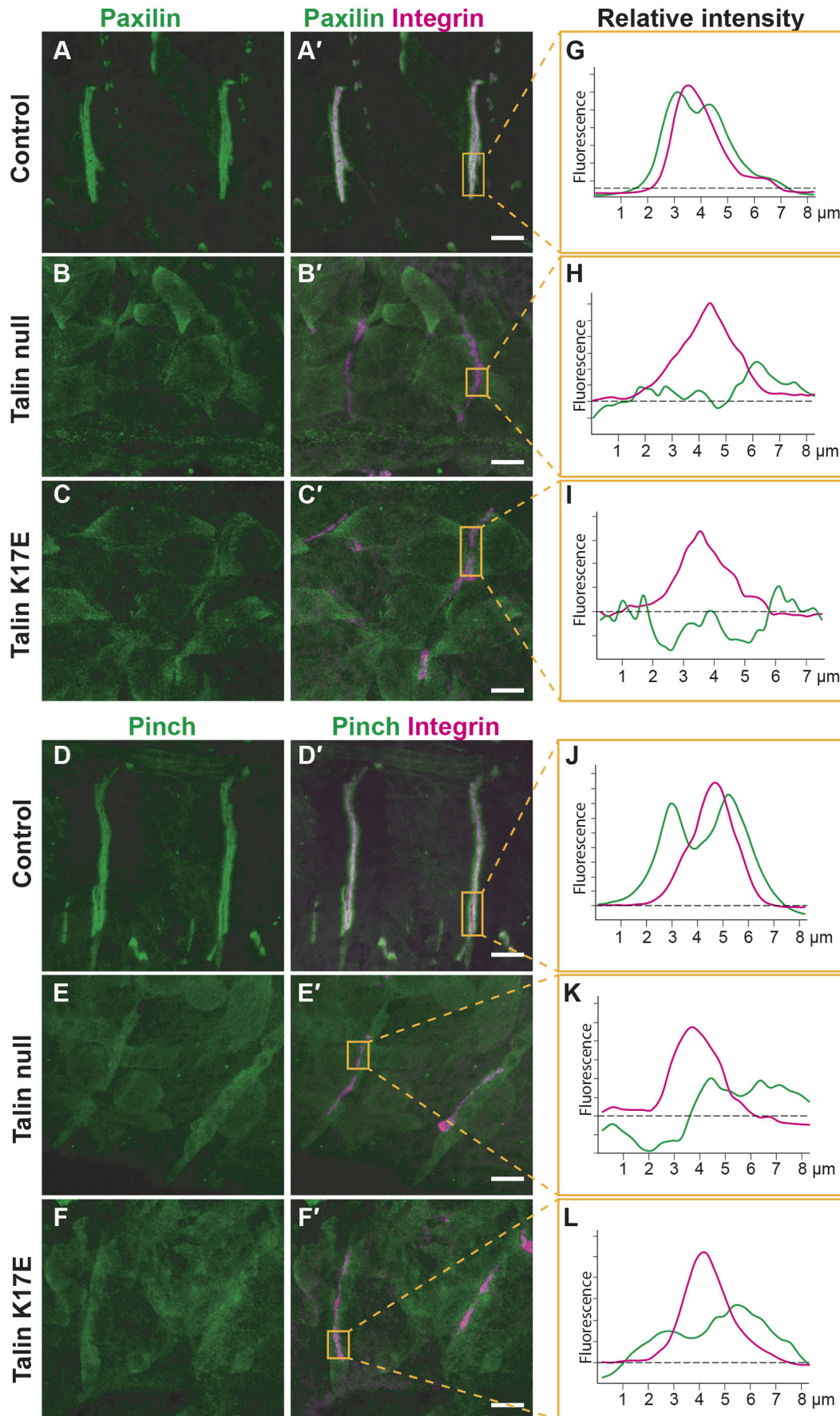


Fig. 4. See next page for legend.

Fig. 4. IAC components recruitment to MTJs is disrupted in K17E mutants. (A–F') Confocal z-stacks of MTJs in stage 17 Talin-null embryos stained for the integrin subunit α PS2 (magenta, A'–F') and IAC components: Paxillin (green, A–C') and PINCH (green, D–F'). (G–L) Average intensity profiles of α PS2 (magenta, G–L), Paxillin (green, G–I) and PINCH (green, J–L) across the widths of the boxed areas indicated in the corresponding images. The dotted line indicates average intensity outside of the MTJ. Scale bars: 10 μ m.

maternally rescued and heterozygous mutant embryos, which both have intact MTJs (Fig. 5L–Q). In embryos containing one mutant copy of either a null or a *rhea*^{K17E} mutant allele, a similar reduction in the levels of Talin at MTJs was observed compared to the wild type (Fig. 5L–N,Q). Furthermore, in maternally rescued null or *rhea*^{K17E} mutant embryos, a substantial and comparable reduction in the levels of Talin at MTJs was observed compared to the wild type (Fig. 5O–Q). Similar localization defects were observed in other tissues; for example, mutant clones of either the null or the *rhea*^{K17E} allele in fly wing discs showed no detectable levels of Talin at the cell cortex, where Talin is usually found (Fig. 5R–T). Taken together, these results show that although Talin containing the K17E mutation is transcribed and translated, it is not properly recruited to or maintained at the cell cortex.

Talin head recruitment to sites of adhesion is regulated by Rap1 through binding to the F0 domain

Based on the effects of the K17E mutation on Talin localization, we hypothesized that the direct binding between Rap1 and the F0 domain of the Talin head plays an important role in regulating the recruitment and/or maintenance of Talin at sites of adhesion. We previously showed that a construct containing the entire Talin head domain (F0, F1, F2 and F3), fused to GFP (TalinHead::GFP), localized efficiently to sites of adhesion at MTJs and was enriched ~3-fold at the MTJs compared to the background staining (Fig. 6A,G; Tanentzapf and Brown, 2006; Tanentzapf et al., 2006). As in previous studies, TalinHead::GFP was also observed in the nucleus, which our past work showed was not a functionally significant localization, but rather an artefact of overexpression of the GFP-tagged fusion protein (Ellis et al., 2011; Tanentzapf et al., 2006). Co-expression of a dominant-negative version of Rap1 (see Materials and Methods; Ellis et al., 2013) reduced the localization of TalinHead::GFP in MTJs to near background levels (Fig. 6B,G). In contrast, co-expression of a constitutively active version of Rap1 (see Materials and Methods; Ellis et al., 2013) enhanced the recruitment of TalinHead::GFP to MTJs by nearly a factor of four compared to background staining (Fig. 6C,G). In comparison to these results, a construct containing a fusion of GFP with the F2 and F3 domains of the Talin head (F2F3::GFP), which contains the integrin binding site but lacks the Rap1-binding F0 domain, localized poorly to MTJs and was enriched by a factor of only ~1.7 compared to background staining at the MTJs (Fig. 6D,H). Importantly, co-expression of either a dominant-negative or a constitutively active version of Rap1 did not change the recruitment of F2F3::GFP (Fig. 6E,F,H), in line with what would be expected of a construct that lacks the direct binding site between Talin and Rap1. These experiments suggest that, in the context of the Talin head domain, the direct binding to Rap1 plays an important function in its recruitment and/or maintenance at sites of adhesion.

Full-length Talin recruitment to sites of adhesion is regulated by Rap1 through binding to the F0 domain

Next, the ability of Rap1 to recruit full-length wild-type and K17E mutant Talin was analysed. Since embryos expressing only the K17E mutant Talin exhibit severe muscle defects that could

interfere with the interpretation of the data, these experiments were performed using zygotic *rhea*^{K17E} mutants, where the embryos still express some maternally provided wild-type Talin during early embryogenesis. This prevents a muscle phenotype from developing until late embryogenesis (see Materials and Methods; Tanentzapf and Brown, 2006). In wild-type embryos, enrichment of full-length Talin was observed at sites of integrin-mediated adhesion at MTJs (Fig. 6I,O). Expression of a dominant-negative version of Rap1 (see Materials and Methods; Ellis et al., 2013) reduced the localization of full-length wild-type Talin at MTJs by ~27% (Fig. 6J,O). In contrast, expression of a constitutively active version of Rap1 (see Materials and Methods; Ellis et al., 2013) enhanced the recruitment of full-length wild-type Talin to MTJs by ~25% compared to background staining (Fig. 6K,O). In zygotic *rhea*^{K17E} mutants, a small amount of Talin was recruited to MTJs, which could represent the maternally provided component of Talin in these embryos (Fig. 6L,P). Expression of a dominant-negative version of Rap1 slightly reduced the levels of Talin present at the MTJs, in line with the hypothesis that the protein we were detecting was the residual maternally provided Talin (Fig. 6M,P). Importantly, expression of the constitutively active version of Rap1 did not change the levels of Talin detected at the MTJs of zygotic *rhea*^{K17E} mutants. This would be the expected outcome if additional recruitment of Talin to sites of adhesion required the direct binding of Rap1 to Talin, as the majority of the Talin present during late embryogenesis in *rhea*^{K17E} mutant embryos is the zygotically transcribed Talin K17E protein that cannot bind Rap1 directly (Fig. 6N,P). Taken together, our data supports the hypothesis that the direct binding of the Talin F0 domain to Rap1 recruits and/or maintains full-length Talin at sites of integrin-based adhesions.

DISCUSSION

Here, we present a biochemical, genetic and phenotypic analysis of the role of direct binding of the F0 domain of Talin to Rap1 in *Drosophila*. Our work suggests that, at least in the context of the simplified adhesion complex of *Drosophila*, direct binding between Talin and Rap1 is functionally important for many biological processes. The key role played by Rap1 in controlling integrin-mediated adhesion is extensively documented in the literature (Boettner and Van Aelst, 2009; Bos et al., 2003). Our analysis adds a new function to the repertoire, whereby Rap1 can regulate cell–ECM adhesion *in vivo*. This mechanism involves an interaction between Rap1 and Talin, which as a direct binding partner of integrins, lies at the heart of the integrin-adhesion complex, and provides Rap1 with a powerful and rapid means of controlling adhesion during the life of an organism.

Although Rap1 has been known as a major regulator of integrin-based adhesion for a long time, the idea that it binds to Talin directly is relatively recent. In *Drosophila* Rap1 plays diverse roles, and in particular is an essential regulator of cell–cell adhesion. Flies completely lacking Rap1 die during the first stages of embryonic development and exhibit severe cellular polarity defects that prevent the development of complex tissue (Knox and Brown, 2002; Choi et al., 2013). Importantly, Rap1 has also been previously implicated in regulating cell–ECM adhesion in *Drosophila*, although the mechanisms remain elusive (Shirinian et al., 2010; Huelsmann et al., 2006; Ellis et al., 2013). Initial indications of a possible direct binding of Talin and Rap1 came from solving the structure of the vertebrate talin 1 F0 domain, which revealed that the F0 domain exhibited structural similarity to the Ras-binding site in RalGDS (Goult et al., 2010). Subsequent studies carried out in both *Dictyostelium* and mammalian cell culture confirmed this direct

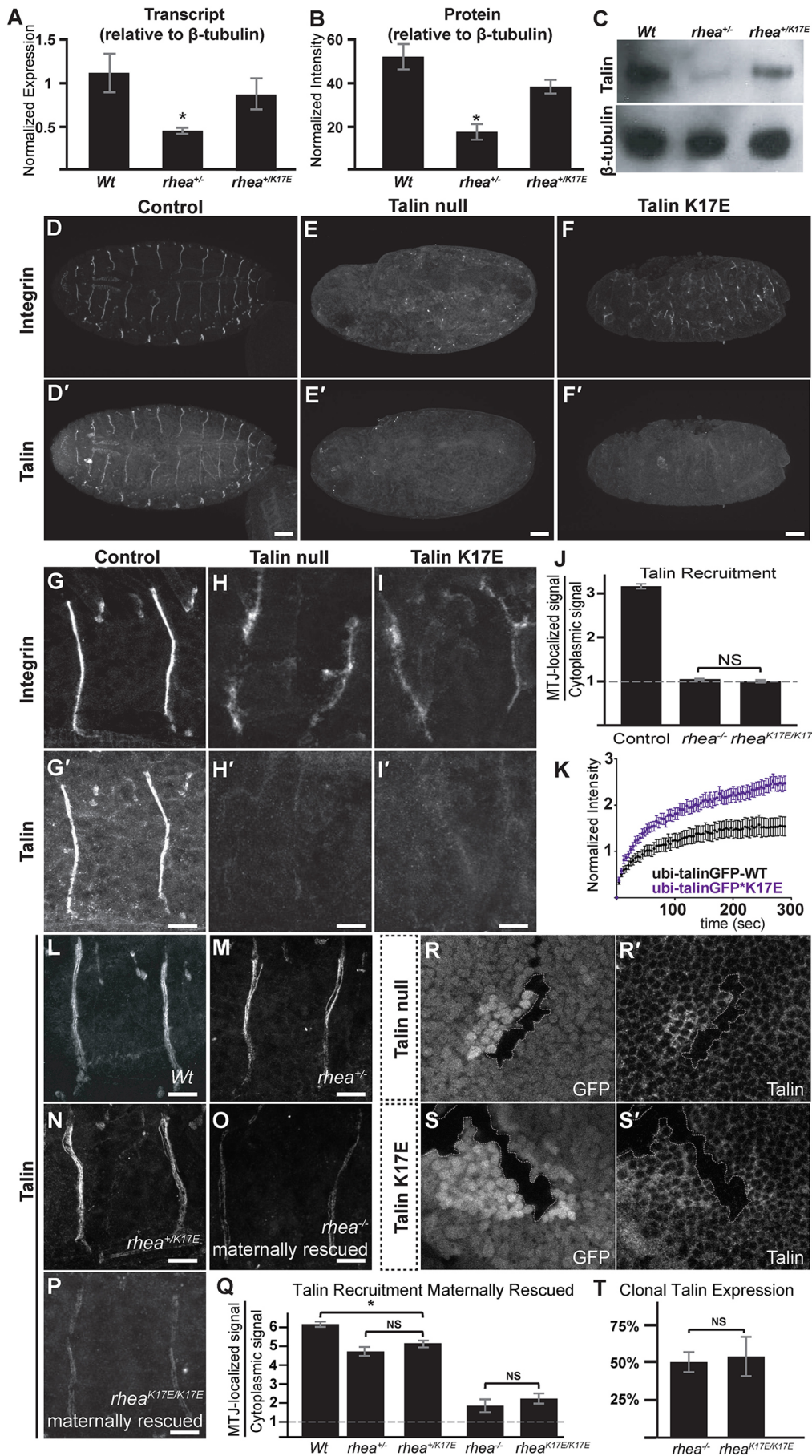


Fig. 5. Talin K17E recruitment. Relative Talin expression levels determined via (A) qPCR and (B,C) western blot analysis of wild-type (*Wt*), *rhea*^{+/-} and *rhea*^{+K17E} heterozygote flies. (D-F') Representative confocal images of whole-mount stage 17 embryos stained for the integrin subunit α PS2 (D-F) or Talin (D'-F'). (G-I) and integrin (G'-I') recruitment at MTJs in control (G,G'), Talin-null (H,H') and K17E embryos (I,I'). (J) Relative localization of Talin to MTJs. (K) Mean recovery intensity of GFP-tagged Talin (black) and GFP-tagged Talin K17E (purple) over time from bleached embryonic MTJs presented with 95% confidence intervals (see Materials and Methods). (L-Q) Zygotic and maternal Talin expression in control (*Wt*, L), Talin heterozygote (*rhea*^{+/-}; M), K17E heterozygote (*rhea*^{+K17E}; N), as well as Talin null (*rhea*^{-/-}) (O) and K17E (*rhea*^{K17E/K17E}) (P) both maternally rescued. (Q) Relative localization of zygotic and maternal Talin to MTJs. (R-T) Clonal analysis of Talin mutants in the wing disc. Talin-null clones (R') and K17E clones (S') are located by the absence of GFP (R,S). (T) Staining intensity inside the clone relative to average intensity outside of the clone. Relative localization was determined by averaging results from 3 MTJs per larva ($n > 10$ per genotypes or clones). Error bars for A, J, K, Q and T represent s.e.m.; error bars for B are s.d. * $P < 0.05$; NS, no significance. Scale bars: 50 μ m (D-F'), 10 μ m (G-P).

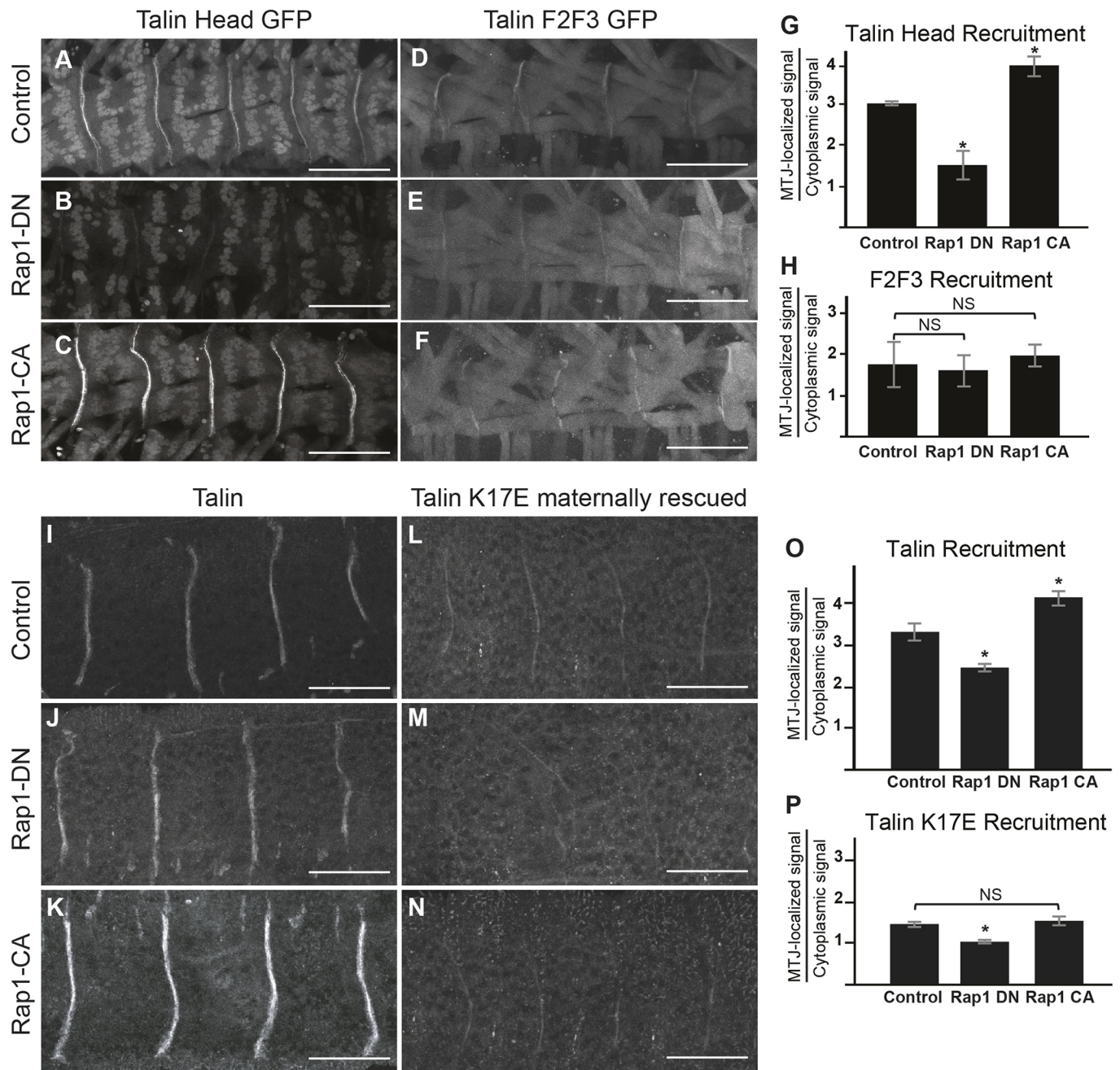


Fig. 6. Rap1 modulation of Talin recruitment requires K17. (A–F) Muscles in live wild-type stage 17 embryos ubiquitously expressing the GFP-tagged Talin head (Talin Head GFP, A–C) or the Talin version deleting the most N-terminal region, which includes the Rap1-binding domain (Talin F2F3 GFP; D–F). Talin head or F2F3 recruitment to the MTJs was assessed in control embryos (A,D) and embryos either expressing a dominant-negative form of Rap1 (Rap1-DN, B,E) or a constitutively active form (Rap1-CA, C,F). (G,H) Relative localization of GFP-tagged Talin Head (G) and Talin F2F3 (H) to MTJs in control, and Rap1-DN- and Rap1-CA-expressing embryos. (I–K) Zygotic and maternal Talin expression in control (I,K), and embryos expressing either Rap1-DN (J) or Rap1-CA (K). (L–N) Zygotic and maternal Talin expression in K17E (*rhea*^{K17E/K17E}) control (L), and maternally rescued K17E embryos (*rhea*^{K17E/K17E}) expressing Rap1-DN (M) or Rap1-CA (N). (O) Relative localization of zygotic and maternal Talin to MTJs in control, and Rap1-DN- and Rap1-CA-expressing embryos. (P) Relative localization of zygotic and maternal Talin to MTJs in control, and Rap1-DN- and Rap1-CA-expressing K17E embryos. Relative localization was determined by averaging results from three MTJs per larva ($n > 10$ per genotypes). Error bars represent s.e.m. * $P < 0.05$; NS, not significant. Scale bars: 50 μ m.

binding and showed that it is functionally important (Plak et al., 2016; Zhu et al., 2017). Two key functional observations have emerged from these studies: first, that the binding of Talin to Rap1 is low affinity and, second, that the binding of Talin to Rap1 regulates Talin recruitment to the membrane (Plak et al., 2016; Zhu et al., 2017). Our work confirms and builds upon these previous observations. The phenotype observed when we introduce a

mutation in Talin that blocks Rap1 binding is indistinguishable from that observed in a null mutation that completely abolishes Talin function. Our FRAP and localization studies suggests that the strong phenotype caused by loss of direct binding of Talin to Rap1 can be explained by a disruption in the ability of the mutant Talin to be localized to and/or be maintained at sites of integrin-mediated adhesion. This result is somewhat surprising because Talin has

multiple means of localizing to sites of integrin-mediated adhesion, including two integrin-binding sites, a binding site for the RAP1-binding scaffolding protein RIAM, a phosphatidylinositol 4,5-bisphosphate (PIP2) interaction domain, as well as multiple other domains that interact with other components of the adhesion complex (Klapholz and Brown, 2017). It thus appears that, at least in certain contexts, the interaction with Rap1, although weak in nature, is of particular importance for controlling Talin localization. Previous work has suggested that the direct interaction between Talin and Rap1 is strengthened when Rap1 is anchored to the membrane (Zhu et al., 2017). In this model, Rap1 localization to sites of adhesion creates a microenvironment that favours the recruitment and/or maintenance of Talin (Zhu et al., 2017). This model is very compatible with our findings and helps explain earlier results that defined a role for Rap1 in regulating integrin-mediated adhesion in flies (Shirinian et al., 2010; Huelsmann et al., 2006; Ellis et al., 2013).

The direct binding of Rap1 to Talin also fits very well with our earlier work on Talin autoinhibition (Ellis et al., 2013). We previously showed that Talin that is unable to undergo autoinhibition localizes to the membrane independently of the presence of RIAM (Ellis et al., 2013). This suggested the existence of a RIAM-independent mechanism for Talin localization. Intriguingly, previous studies show that the F2F3 domain of Talin contains a conserved RIAM-binding site (Yang et al., 2014). Our results show that although the F2F3 domain of Talin localizes to sites of adhesion, it does so less efficiently than the full Talin head domain (containing F0–F3). Furthermore, in contrast to the full-length Talin head construct, the recruitment of a construct containing only the F2F3 domain was not efficiently modulated by the presence of constitutively activated Rap1. This indicates that the main way Rap1 controls the recruitment of the Talin head domain to sites of integrin-mediated adhesion is through direct binding to Talin rather than indirect binding through RIAM. Support for these conclusions comes from studies in mice that showed that RIAM was dispensable in most tissues for Talin localization and integrin activation (Stritt et al., 2015). Therefore, it appears that in flies, and possibly, in some tissues in mice, the direct interaction of Talin with Rap is both necessary and sufficient for recruitment and/or maintenance of Talin at sites of integrin-mediated adhesion, and that this applies to both autoinhibited and non-autoinhibited Talin.

Our data suggests that a Talin mutant that is unable to bind directly to Rap1 is transcribed, translated and folds normally, but is unable to function. While we favour a model wherein the strong phenotype we observe is due to an inability to localize and/or maintain Talin at sites of adhesion, we cannot discount several other alternative hypotheses. For example, it could be that, in addition to disrupting the interaction of Talin with Rap1, the K17 mutation also disrupts the interaction of Talin with proteins other than Rap1. Possible candidates for such additional interactions with the Talin F0 domain are other Ras GTPases encoded by the *Drosophila* genome. However, the possible roles of fly GTPases were explored in the context of a genome wide analysis that analysed, among other phenotypes, disruption to integrin-based myotendinous junctions (Schnorrer et al., 2010). This analysis failed to identify a role for additional Ras GTPases in integrin-mediated muscle tendon attachment. Additionally, while our experiments show direct F0–Rap1 interactions *in vitro* they do not address the specificity or selectivity of the interaction. The overall weak binding between Talin F0 and Rap1 makes it difficult to illustrate this interaction *in vivo*, which leaves the possibility of indirect binding or that

additional, partially redundant, factors are involved. Furthermore, in previous work we showed that making a ‘headless’ version of Talin by deleting the entire Talin head (residues 1–448), while leaving the rest of Talin intact results in a Talin protein that is completely non-functional, but that can still partially localize to sites of adhesion (Ellis et al., 2014). Given this result, it is curious that mutating the single K17 residue in the Talin head completely abolishes Talin localization. One possible explanation is that the headless version of Talin was expressed from a ubiquitous promoter in an exogenous rescue construct, in contrast to the CRISPR approach we used here. Another possible explanation is that the headless Talin was tagged with GFP, and was detected using an extremely sensitive GFP-specific antibody in contrast to the Talin-specific antibody we used to detect the K17 mutant Talin in the present study. Nonetheless, these results hint at the complicated network of positive and negative reinforcement cues that operate on Talin to regulate its localization to the membrane.

It remains to be fully established whether the important role of direct binding between Talin and Rap1 is conserved in vertebrates. The higher complexity of integrin-based adhesions in vertebrates provides alternative regulatory mechanisms that can mask the sort of dramatic phenotypic effects observed in disruptions of the simpler integrin-based adhesions found in the fly. Consistent with this idea is the recent analysis of mice containing a mutation in Talin designed to block the interaction of the F0 domain of Talin with Rap1 (Lagarigue et al., 2018). That work shows that, at least in the context of blood platelets, the direct interaction between the F0 domain and Rap1 is not essential for integrin activation. This suggests two possible differences between the fly and vertebrate integrin-based adhesions, the first is that, in vertebrates, Talin can be recruited to the membrane by multiple, Rap1-independent, mechanisms, and the second is that, in vertebrates, Rap1 binds to Talin directly through another interaction that does not involve the F0 domain and which is not conserved in flies. Nonetheless, by showing in the fly that Rap1 binding is essential for Talin recruitment to sites of adhesion our work raises the possibility that, at least in some contexts in vertebrates, the direct binding of Talin to Rap1 may prove to be functionally important.

MATERIALS AND METHODS

Molecular biology

The generation of ubi-talinGFP was previously described (Yuan et al., 2010). To make the pUbi-talinEGFP*K17E mutant construct, pBS-talinGFP was mutated using the QuikChange Lightning mutagenesis kit (Stratagene). The talinGFP*K17E cassette was sub-cloned into the pUbi63E vector using a strategy similar to that used to generate the WT talinGFP construct (Yuan et al., 2010). The making of pUASp-GFP-TalinHead, F2F3, UAS Rap1CA, and UAS Rap1 DN was described previously (Tanentzapf et al., 2006; Ellis et al., 2013). The generation of the K17E mutant described here (Fig. 2) is based on a modified version of the previous protocols (<http://flycrispr.molbio.wisc.edu> and Gratz et al., 2014). The following target sequences were respectively used for S1 and S2: 5'-GAAACCACCCCAAAGCGCAAGG-3' and 5'-GATAAACAGTCCAT-ATTGCTGG-3'. The first homology arm was directly amplified from genomic *Drosophila* DNA using the following primers: 5'-GCACACC-TGCGATCTCGCTTGTTCGGCACATACGAGC-3' and 5'-GATTCA-CCTGCGCACTATTACAATTTGAGCTTATGTTTTTAAGA-3'. The second arm containing the K17E mutation as well as the second homology arm was amplified from pUBiK17E plasmid using the following primers: 5'-AGGAGCTCTTCATATTAATGAGGAAATT-CGTTGAAATTT-3' and 5'-ACGTGCTCTTCaGACGTCGCCAAAGT-CCAGAGTGA-3'. Both arms were then seamlessly cloned in the pHD-DsRed (Gratz et al., 2014), respectively, using the Aar1 and Sap1 sites to generate the double-stranded donor DNA. S1 and S2 targeting

gRNAs were cloned by annealing the corresponding target sequence oligonucleotides into the pU6-BbsI-chiRNA plasmid (Gratz et al., 2014) via the BbsI restriction sites. The CRISPR injection mix containing the double-stranded donor plasmid (500 ng/μl) along with both targeting plasmids (100 ng/μl each) was sent to Bestgene Inc. for injection. K17E transgenic flies were identified by eye colour screening using the DsRed gene included in the donor DNA. This visible eye marker was then excised by crossing K17E mutants to P{y[+mDint2]=Crey}1b flies expressing Cre recombinase and leaving behind a residual 34 bp residual loxP site. The resulting K17E mutant was then recombined in an FRT2A background and sequenced using standard techniques (Fig. 2D).

Fly stocks and genetics

Unless otherwise specified all experiments were performed in mutant background such that wild-type maternal contributions of Talin were eliminated using the *rhea*^{79a} or *rhea*^{K17E} alleles and the dominant female sterile technique (Chou and Perrimon, 1996). Females of the genotype *yw*, *hs-Flp/+*; *rhea*^{79a} or K17E, FRT2A/OvoD1, FRT2A were subjected to a heatshock regime during the larval stages to generate mosaic germline in order to give rise to *rhea* mutant oocytes. Virgins were then crossed to *rhea*^{79a} or *rhea*^{K17E}/TM6b, *dfd-GMR-nvYFP* males. Embryos without the fluorescent balancer were selected for analyses. For all FRAP experiments, *ubi-talinGFP-WT* and *ubi-talinGFP-K17E* constructs were heterozygous and expressed in a *w¹¹¹⁸* background. In the case where UAS-driven transgenes were utilized, comparable controls were taken from flies expressing the UAS transgene, but without the Gal4 driver. Dominant-negative and constitutively active Rap1 (Ellis et al., 2013) were driven in the muscles using the *mef2-GAL4* driver.

Confocal immunofluorescence imaging and image analysis

Embryos were fixed and stained according to standard protocols; Tanentzapf and Brown (2006). Antibodies used were against the following proteins: MHC (mouse, 1:200; Dan Kiehart, Duke University, Durham, NC), PINCH (rabbit, 1:1000; Mary Beckerle, Huntsman Cancer Institute, UT), Talin (rabbit, 1:500), αPS2 (rat, 1:100; 7A10), paxillin (rabbit, 1:1000) (Yagi et al., 2001), tiggrin (mouse, 1:500; Liselotte Fessler, UCLA, CA), PINCH (rabbit, 1:1000; Mary Beckerle, University of Utah) and GFP (rabbit, 1:1000; A6455, Invitrogen). Fluorescently conjugated Alexa Fluor 488, Cy3 and Cy5 secondary antibodies were used at 1:400 dilution (Molecular Probes). Images were collected using an Olympus FV1000 inverted confocal microscope. For all micrographs of whole embryos, or of MTJs, *z*-stacks were assembled from 8–12 0.5 μm confocal sections. Statistically significant differences were assessed by means of a two-tailed Student's *t*-tests in all cases, except when we sought to compare between multiple constructs, where one-way ANOVA was used. Statistical analysis was carried out using Prism4 software (GraphPad, La Jolla, CA). For intensity traces across MTJs, the ImageJ plot profile tool was used to determine the average signal intensity across the boxed area indicated on the images. Intensity curves were obtained from unprocessed greyscale images so that first the peak intensity of each channel across the area of interest was set as 100%. Each curve was then normalized to the average intensity measured outside of the MTJ.

FRAP

Stage 17 embryos were collected and prepared for FRAP as described previously (Yuan et al., 2010). Briefly, embryos were collected from grape juice plates, dechorinated in 50% bleach for 4 min, washed with PBS and mounted onto glass slides in PBS. Photo-bleaching was performed using a 473 nm laser at 5% power with the Tornado scanning tool (Olympus) for 2 s at 100 ms per pixel. Fluorescence recovery was recorded over 5 min at 1 frame every 4 s. To control for the muscle twitching in and out of focus, multiple regions of interest (ROIs) were selected in non-photobleached regions; only samples for which intensities within control ROIs remained steady throughout the FRAP experiment were used. The mobile fraction and statistical tests were performed using Prism 5 software.

Western blot analysis and qPCR

For the quantitative real-time PCR (qPCR) total RNA was isolated from whole flies using TRIzol. A total of 0.5 μg total RNA was converted into

cDNA using the qScript cDNA synthesis kit (Quanta Biosciences). Subsequently, qPCR was performed using iQ SYBR Green Supermix (Biorad). Talin mRNA levels were averaged between three independent experiments performed four times and normalized to β-tubulin expression. Primers used for Talin were located 3' to the K17E mutation and were as follow 5'-GCCAGAACAATACCTTTGGGTCG-3' and 5'-AACTGGGC-ATTTCGCTGGAA-3'. β-tubulin expression was determined using the primer pair 5'-ATCATCACACACGGACAGGA-3' and 5'-GAGCTG-GATGATGGGGAGTA-3'. For western blot analysis, protein samples were homogenized in 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.5% Triton and EDTA-free complete protease inhibitor cocktail (Roche) and cleared by centrifugation at 16,000 g for 30 min at 4°C. After the addition of SDS sample buffer, samples were heated for 5 min at 100°C and were resolved using a 7% gel. Primary antibodies used were: 1:500 mouse anti-talin [E16B, Developmental Studies Hybridoma Bank (DHSB)] and 1:500 β-tubulin (E7, DHSB). Secondary antibodies used were: 1:3000 anti-mouse-IgG conjugated to HRP (Biorad). Chemiluminescent substrate (Clarity Western ECL Biorad) was applied according to the manufacturer's instruction and blots were exposed to X-ray film. Triplicate samples were quantified after background subtraction and normalization to β-tubulin bands levels.

NMR

cDNA encoding fly Talin residues 1–87 (F0) was synthesized by PCR using a fly *talin* cDNA as template, cloned into the expression vector pet-151, and expressed in *E. coli* BL21 STAR (DE3) cultured in 2xM9 minimal medium for preparation of isotopically labelled samples for NMR. Recombinant His-tagged Talin polypeptides were purified by nickel-affinity chromatography following the standard protocol. The His tag was removed by cleavage with AcTEV protease (Invitrogen), and the proteins further purified by anion exchange. The murine Rap1 isoform Rap1b (residues 1–166) was expressed from a pTAC vector in *E. coli* strain CK600K. Cultures were grown at 37°C to an optical density at 595 nm (OD₅₉₅) of 0.8 when they were induced with 200 μM IPTG and then cultured at 18°C overnight. Protein was purified by ion exchange, followed by gel filtration.

NMR spectra were measured at 298 K using a Bruker AVANCE DRX 600 spectrometer equipped with a CryoProbe. NMR spectra were obtained using a Bruker AVANCE III 600 MHz spectrometer equipped with a CryoProbe. Experiments were performed at 298 K in 20 mM sodium phosphate buffer pH 6.5, 50 mM NaCl, 2 mM DTT with 5% (v/v) ²H₂O. Proton chemical shifts were referenced to external 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). The ¹⁵N and ¹³C chemical shifts were referenced indirectly using recommended gyromagnetic ratios (Wishart et al., 1995). Spectra were processed with TopSpin (Bruker) and analysed using ANALYSIS (Vranken et al., 2005).

Ligand binding was evaluated from ¹H,¹⁵N-HSQC chemical shift changes using 50 μM ¹⁵N-labelled F0. Rap1 was added up to a 7:1 Rap1:F0 ratio. 3D HNCO, HN(CA)CO, HNCA, HN(CO)CA, HNCACB and HN(CO)CACB experiments were used for the sequential assignment of the backbone NH, N, CO, Cα and Cβ resonances as described previously (Skinner et al., 2015). The backbone resonance assignments of fly Talin F0 (1–87) have been deposited in the BioMagResBank with the accession number 26884.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: D.C., E.L., B.T.G., G.T.; Methodology: D.C., A.H., V.S., W.M.C., Q.A.X., B.T.G., G.T.; Software: B.T.G.; Validation: D.C., Q.A.X., B.T.G., G.T.; Formal analysis: D.C., A.H., V.S., W.M.C., Q.A.X., B.T.G.; Investigation: D.C., W.M.C., B.T.G.; Resources: D.C., B.T.G., G.T.; Data curation: D.C., A.H., V.S., W.M.C., Q.A.X., E.L., B.T.G.; Writing - original draft: D.C., G.T.; Writing - review & editing:

D.C., A.H., B.T.G., G.T.; Visualization: D.C.; Supervision: D.C., A.H., B.T.G., G.T.; Project administration: D.C., B.T.G.; Funding acquisition: B.T.G., G.T.

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Data availability

The backbone resonance assignments of fly Talin F0 (1–87) have been deposited in the BioMagResBank with the accession number 26884.

Supplementary information

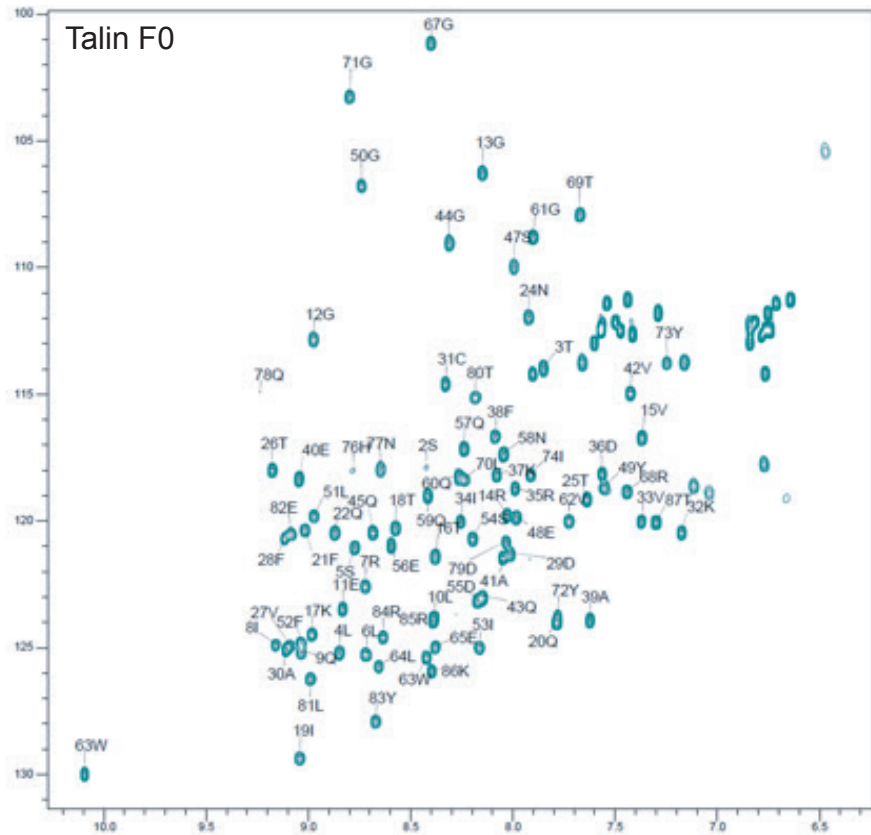
Supplementary information available online at <http://jcs.biologists.org/lookup/doi/10.1242/jcs.225144.supplemental>

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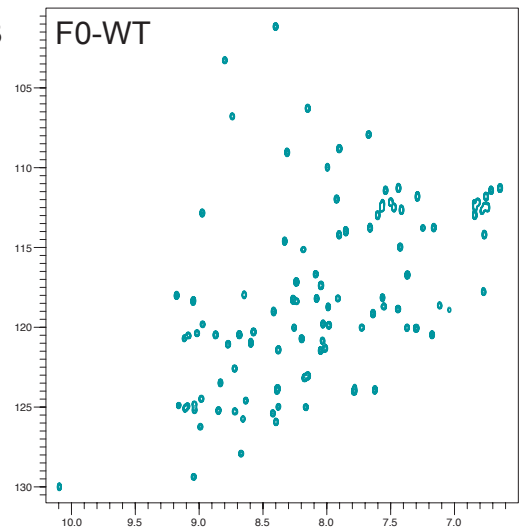
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A



B



C

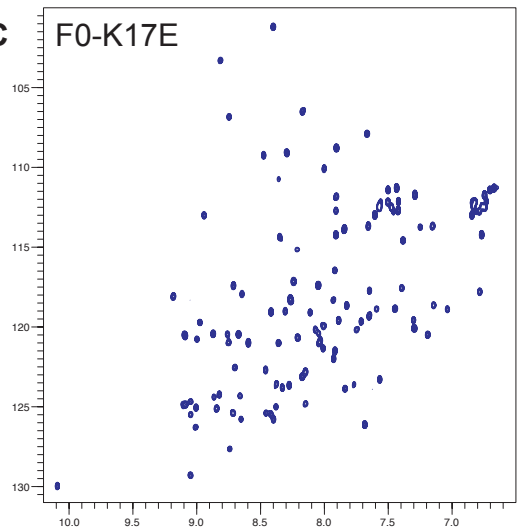


Figure S1.

(A) Assigned ^1H , ^{15}N HSQC spectrum of fly Talin F0 (residues 1-87). (B-C) ^1H , ^{15}N HSQC spectra of talin F0 (B) wildtype and (C) K17E.

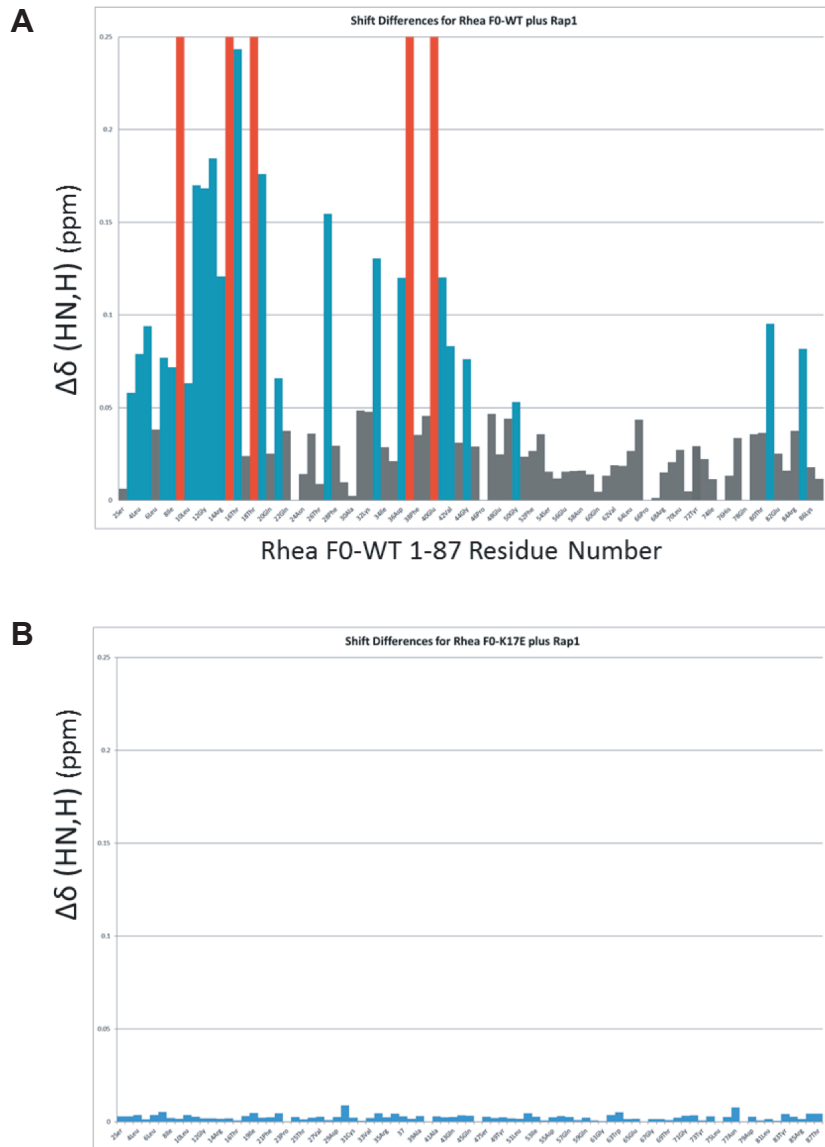


Figure S2.

(A-B) Weighted shift map obtained from the ^1H , ^{15}N HSQC spectra of the talin F0 domain with the addition of Rap1b. (A) Wildtype F0 and (B) K17E F0.