Focal adhesion kinase is not required for integrin function or viability in *Drosophila*

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Summary

The mammalian focal adhesion kinase (FAK) family of non-receptor protein-tyrosine kinases has been implicated in controlling a multitude of cellular responses to the engagement of cell-surface integrins and G-protein-coupled receptors. The high level of sequence conservation between the mammalian proteins and the *Drosophila* homologue of FAK, Fak56, suggested that it would have similar functions. However, we show here that *Drosophila* Fak56 is not essential for integrin functions in adhesion, migration or signaling in vivo. Furthermore, animals lacking Fak56 are viable and fertile, demonstrating that Fak56 is not essential for other developmental or physiological functions. Despite this, overexpressed Fak56 is a potent inhibitor of integrins binding to the extracellular matrix, suggesting that Fak56 may play a subtle role in the negative regulation of integrin adhesion.

Key words: *Drosophila*, FAK, Integrins, Signal transduction

Introduction

Focal adhesion kinase (FAK) is a eukaryotic non-receptor protein tyrosine kinase (PTK) that is implicated in the regulation of a multitude of cellular responses, from the engagement of cell surface integrins and G-protein-coupled receptors, to adhesion, migration, differentiation, proliferation and survival (for reviews see Gelman, 2003; Parsons, 2003). FAK is the founder member of a family of non-receptor PTKs, which include FAK and the related kinase Pyk2 (also known as CAKβ, RAFTK, FAK2 and CADTK) in mammals (Avraham et al., 1995; Guan and Shalloway, 1992; Hanks et al., 1992; Herzog et al., 1996; Lev et al., 1995; Sasaki et al., 1995; Schaller et al., 1992; Yu et al., 1996). FAK is widely expressed and highly tyrosine phosphorylated throughout embryogenesis, while Pyk2 is more restricted in its expression (Avraham et al., 2000). Major structural features of FAK family PTKs include a centrally located kinase domain, flanked by an N-terminal FERM domain, proline-rich regions, a C-terminally located FAT domain and multiple tyrosine phosphorylation sites, including the important autophosphorylation site, tyrosine 397 (tyrosine 402 in Pyk2) (Gelman, 2003; Parsons, 2003). Studies in mammalian cells have shown that activation of FAK is initiated by phosphorylation of tyrosine 397, creating a binding site for the SH2 domain of Src family tyrosine kinases (Calalb et al., 1995). The formation of the FAK-Src signaling complex results in further tyrosine phosphorylation of FAK and other associated proteins (e.g. $p130^{Cas}$ and paxillin) and subsequently the recruitment of multiple cellular components, including both other focal-adhesion-associated proteins (such as talin) and signaling proteins (e.g. Grb2, Crk, Nck, PI3K, GRAF) (for reviews, see Gelman, 2003; Parsons, 2003).

FAK localization to focal adhesions is mediated by the C-terminally located focal adhesion targeting (FAT) domain (Hildebrand et al., 1993; Hildebrand et al., 1995). Additionally, the N-terminal region of FAK has been proposed to bind directly to the cytoplasmic tail of β-integrins, which are thought to be the major regulators of FAK activity (Schaller et al., 1995), and it has recently been reported that the cytoplasmic tail of β1-integrin stimulates FAK activity in vitro (Cooper et al., 2003).

Integrins are the major family of cell surface receptors that link the extracellular matrix (ECM) to the actin cytoskeleton (Watt, 2002). They are heterodimeric glycoproteins, which function as receptors for a variety of ECM proteins such as fibronectin, collagen and laminin. In addition to providing this structural link of cell adhesion, integrins also activate many intracellular signaling pathways and influence many intracellular events that play key roles during, for example, development and immune responses (Hynes, 2002). The mechanism that links integrin clustering to FAK activation and the role of FAK in integrin signaling pathways has been intensively studied in mammalian cells in culture, leading to the identification of a number of diverse pathways downstream of FAK, but the significance of these in the intact animal is as yet unclear.

An essential role for FAK in mammals has been demonstrated by genetic studies in mice. The mouse FAK

knockout (*Ptk2*$^{-/-}$ – Mouse Genome Informatics) dies early in embryogenesis (Ilic et al., 1995), with defects in mesoderm development that are similar to those caused by the knockout of fibronectin (George et al., 1993). Studies on FAK-null cells derived from these mice have indicated that rather than being involved in the assembly of integrin adhesive junctions, FAK may be involved in their remodeling (Webb et al., 2004), a process critical for cells to migrate. Thus, *Ptk2*$^{-/-}$ fibroblasts exhibit a rounded morphology, with an increased number of focal contact sites and decreased rates of cell migration (Ilic et al., 1995). Interestingly, inhibition of Rho signaling can partially reverse these morphological and motility defects (Chen et al., 2002), while v-Src transformation of *Ptk2*$^{-/-}$ fibroblasts rescues the integrin-stimulated motility defects as well as re-introduction of FAK itself (Hsia et al., 2003). Furthermore, studies in *Ptk2*$^{-/-}$ fibroblasts have demonstrated a role for FAK in the organization of the fibronectin matrix (Ilic et al., 2004). This has recently been elegantly shown in vivo, where conditional loss of FAK in the developing dorsal forebrain of mice results in altered basement membrane organization (Beggs et al., 2003).

In contrast to the dramatic phenotypes observed in *Ptk2* mutant mice, *Pyk2* mutant animals are viable with no gross defects in adhesion, instead displaying immune system defects (Guinamard et al., 2000). However, the lack of strong phenotypes in *Pyk2* mutant mice may be misleading, since it is unclear whether the presence of the wild-type *FAK* locus is able to compensate for the lack of Pyk2 in this case. This is an important consideration since it has been shown that the targeting of Pyk2 to β1-integrin-containing focal contacts can rescue the fibronectin-stimulated signaling and motility effects observed in FAK-null cells (Klingbeil et al., 2001).

In order to identify functions of FAK that are conserved in metazoan evolution, we have investigated the role of FAK in *Drosophila*. A single protein with the domain structure characteristics of FAK and Pyk2 is encoded by the *Drosophila* genome (Adams et al., 2000; Fox et al., 1999; Fujimoto et al., 1999; Palmer et al., 1999). This protein, Fak56 (Fak56D – FlyBase), exhibits a high overall amino acid similarity with human FAK. In the fly, Fak56 is ubiquitously expressed with particularly high levels in the developing CNS and muscle (Fox et al., 1999; Fujimoto et al., 1999; Palmer et al., 1999). It is phosphorylated on tyrosine in vivo and this phosphorylation is increased upon plating *Drosophila* cells onto ECM proteins. Overexpression of Fak56 results in lethality when ubiquitously expressed, and when expressed more selectively gives phenotypes such as wing blistering, which are characteristic of loss of integrin function (Palmer et al., 1999). Integrins have been found to be essential for diverse developmental functions in the fly, including mediating strong adhesion between two layers of cells, particularly at muscle attachment sites and between the two surfaces of the developing wing, mediating migration, and regulation of gene expression during differentiation (Bokel and Brown, 2002; Brower, 2003).

In order to use *Drosophila* to learn more about FAK function in vivo, we generated mutants in the gene encoding Fak56 and analyzed their phenotype. Given that mutations in *Ptk2* are lethal in the mouse, we were surprised to find that flies completely lacking Fak56 were viable and fertile. We examined integrin-dependent processes and the development of cells known to have particularly high levels of FAK expression, but did not detect any defects. This indicates that Fak56, contrary to earlier assumptions, is not critically required for fly development or physiology.

Materials and methods

Drosophila stocks

Standard *Drosophila* husbandry procedures were employed. Flies were raised and crossed at room temperature unless otherwise stated. The wild-type strain used was *white*^{1118}. The P{SUPor-P} line KG00304 (#13080) and the transposase source P[*ry*$^{+t7.2}$ Δ2.3](99B) lines (#3612 and #3629) were obtained from the Bloomington stock center. The 5-SZ-3124 and UM-8250-3 lines used to generate Df(2R)ED3716^{CG2} were obtained from the Szeged stock center. The CyOWg-lacZ balancer chromosome was used to genotype embryos. Mutants in the integrin αPS2 subunit, *inflated*^{B4} (Brown, 1994), and the βPS subunit, *myospheroid*^{mj42} (Wilcox et al., 1989) and *myospheroid*^{b43} (Jannuzi et al., 2004), were kindly provided by Dr D. Brower. UAS:Fak56 (Palmer et al., 1999), *Fak56* genomic rescue and UAS:Fak56-GFP (see Data S1 in the supplementary material) transgenic flies were generated by CG and RHP. Mef2-Gal4 and Engrailed-Gal4 were used to drive expression of these UAS constructs. ILK-GFP was used as described previously (Zervas et al., 2001). Overexpression of Fak56 in the primordial midgut was achieved with the 48Y-Gal4 line (Martin-Bermudo et al., 1999). *white*^{1118} were used for all microinjections described.

Generation of *Fak56* mutants

The P{SUPor-P} line [KG00304] with a P-element insertion 5′ to the *Fak56* gene was mobilized using *P[ry*$^{+t7.2}$ *Δ2.3](99B)* as a transposase source. A total of 750 independent excision lines (both viable and non-viable) were established, and subjected to Southern blot analysis using two independent *Fak56* genomic regions as probes. FAK I probe corresponds to a PCR fragment encompassing bp 14-743 in the genomic *Fak56* sequence (with 1 denoting the start of transcription) and the FAK II probe, bp 2300-3008 (Fig. 1B). Primers used were: for FAK I, 5′-GAGCCACCAGTCAAC-3′ and 5′-GCTTTGATGTG-GCTATCA-3′; for FAK II, 5′-GGATTATCACGTTGGGTT-3′ and 5′-AATAATATAGGTCTGAGG-3′.

A 12.8 kb deletion encompassing the *Fak56* locus was generated using FRT recombination between the 5-SZ-3124 and UM-8250-3 P elements according to the DrosDel Isogenic Deficiency kit instructions (Golic and Golic, 1996; Ryder et al., 2004). The resulting *Df(2R)ED3716*^{CG2} mutation was subjected to Southern blot analysis, using two independent *Fak56* genomic regions as probe, and sequence analysis to confirm the breakpoints.

Drosophila DNA isolation and Southern hybridization

Genomic DNA was prepared using standard techniques. Genomic DNA, digested with *Eco*RI (New England Biolabs), was electrophoresed on 1.0% agarose gel and blotted onto Hybond N+ filter (Amersham) using standard techniques. DNA was crosslinked to the filter by UV-exposure. Filters were prehybridized in prehybridization buffer (0.7% SDS, 50% formamide, 5× SSC, 50 mM Na-phosphate buffer pH 7.0, 1% Blocking reagent) for 1-4 h at 42°C prior to incubation with DIG-labeled Fak56 or Spt5 probes at 42°C overnight. After washing, the filters were analyzed by the DIG detection chemiluminescent assay according to manufacturer's recommendations (Roche).

PCR and sequencing

Standard molecular biology protocols were used. PCR over the excision breakpoint was performed on genomic DNA prepared from *Fak56*^{CG1} flies using the primers 5′-CTTTCCACGCCAGTGGTGG-3′ and 5′-GCATAATCATCGGTCAGCATCGG-3′. Similarly, PCR over the deletion breakpoints of *Df(2R)ED3716*^{CG2} was performed as

follows: (1) on the 3′ side using the primers 5′-GCAGGTGCTCTT-GCGGAC-3′ and 5′-TTATGAGTTAATTCAAACCCCAC-3′ and (2) on the 5′ side using the primers 5′-CGTACTTTGGAGTACGAAAT-GC-3′ and 5′-CACACATACGCCACAGAGGGAG-3′. Sequencing data were collected using the BigDye™ Terminator Sequencing kit v2.0 (Applied Biosystems). Sequence was determined on both strands.

Drosophila protein isolation and western blotting

Drosophila embryos were dechorionated in 50% sodium hypochlorite and washed extensively in PBS prior to lysis in lysis buffer (4% SDS, 20% glycerol, 100 mM Tris/HCl pH 6.8, 40% β-mercaptoethanol). Lysates were cleared by centrifugation and protein concentrations determined using the Bio-Rad protein assay. Protein samples were separated on SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). Membranes were blocked in 5% milk (in 1× PBS, 0.1% Tween-20) for 1 h, prior to incubation with primary antibodies overnight, and ECL detection (Amersham Pharmacia Biotech).

Immunostaining and antibodies

Embryos were fixed and immunostained as described previously (Patel, 1994). Rabbit anti-Fak56 was used at 1:1500 (Palmer et al., 1999). Phospho-FAK^{Y397} was used at 1:1000 (Biosource). Mouse monoclonal anti-βPS integrin (CF.6G11) was used at 1:10 (Developmental Hybridoma Bank). Mouse polyclonal anti-Tiggrin (Fogerty et al., 1994) was used at 1:1000 (kind gift from Dr L. Fessler). Mouse monoclonal anti-Talin (Brown et al., 2002) was used at 1:5. Mouse monoclonal anti-α-Tubulin was used at 1:5000 (Sigma). Mouse monoclonal anti-MHC (muscle myosin heavy chain) (Bloor and Kiehart, 2001; Kiehart et al., 1990) was used at 1:5 (kind gift from Dr D. Kiehart). Actin was visualized with Rhodamine-Phalloidin (Sigma). Staining with Rhodamine-Phalloidin required embryo devitelinization in 90% ethanol. Immunolocalization was visualized with fluorescent secondary antibodies (Southern Biotechnology Associates). Imaginal discs were fixed in 4% PFA in PBS for 1 hour on ice, and stained as for the embryos.

Results

Previously a single FAK family PTK, encoded by the *Fak56* gene, has been described in the *Drosophila* genome (Fig. 1A) (Fox et al., 1999; Fujimoto et al., 1999; Palmer et al., 1999). To generate mutations in the *Fak56* gene, we initially performed several P element screens for lethal mutants, and failed to identify a *Fak56* mutant. We therefore screened more directly for deletions of the coding region in both viable and lethal mutant lines. To generate deletions in the *Fak56* gene by imprecise excision, we mobilized a P{SUPor-P} transposon (Roseman et al., 1995) that is inserted 373 bp upstream of the *Fak56* ATG (Fig. 1B), and screened for putative *Fak56* mutant alleles by Southern blot analysis. The breakpoint of one of the alleles identified, *Fak56^{CG1}*, was then precisely mapped via Southern and subsequent PCR and sequence analysis (Fig. 1C,D). This mutant consists of a deletion that removes the entire P-element and 1887 bp of the *Fak56* gene (Fig. 1B). As a result of this deletion, the *Fak56^{CG1}* allele lacks the first 1263 bp of coding sequence, corresponding to the first 421 amino acids of Fak56 (Fig. 1C,D). Importantly, the *Fak56^{CG1}* deletion does not disrupt the adjacent gene at the 5′ end of *Fak56*, *Spt5* (Fig. 1D), since excisions that affect this gene proved to be lethal.

To confirm that we had generated a null allele, expression of the Fak56 protein in *Fak56^{CG1}* mutants was examined using an antiserum directed against the C-terminal part of Fak56 (Palmer et al., 1999). In wild-type embryos, this antiserum

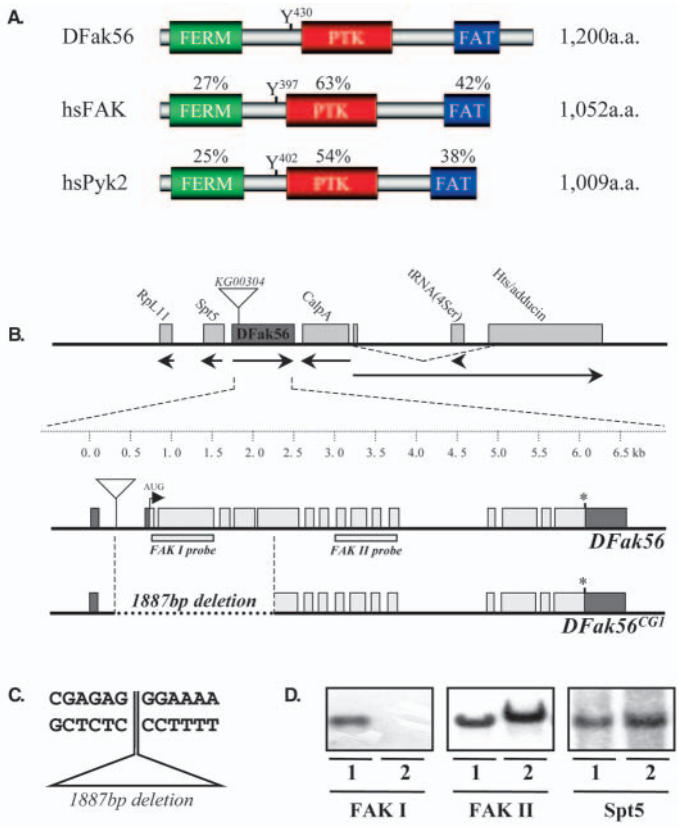

Fig. 1. Molecular organization of the *Fak56* locus and characterization of *Fak56^{CG1}* mutant allele. (A) Schematic comparison of Fak56 with the mammalian FAK family kinases, hsFAK and hsPyk2. Conserved domains are schematized as follows; FERM domain (green), protein tyrosine kinase (PTK) domain (red) and FAT domain (blue). Percentage amino acid identity of FAK domains relative to the *Drosophila* Fak56 is indicated. (B) Genetic analysis of *Fak56*. Top panel: the *Fak56* locus is depicted together with the surrounding genes (indicated as gray boxes). The arrows below indicate the orientation of *Fak56* and the surrounding genes. The location of the P-element insertion used in the excision screen, P{SUPor-P}KG00304 is shown by an inverted triangle. Middle panel: the deduced structure of the approximately 7 kb *Fak56* transcription unit is shown in expanded form. The 16 exons are represented by gray boxes. The first AUG initiation codon at the 5′ end of the gene is indicated by a flag, and the TAA termination codon at the 3′ end is marked by an asterisk. The horizontal gray bars represent the genomic probes employed to identify *Fak56* mutants. The FAK I probe corresponds to bp 14-743 and the FAK II probe, bp 2300-3008 (where bp 1 corresponds to the A of the ATG start codon of *Fak56*). Lower panel: by mobilization of transposon P{SUPor-P}KG00304 373 bp upstream of the *Fak56* ATG start codon, deletion *Fak56^{CG1}*, which deletes the ATG start codon and eliminates 1263 bp of the *Fak56* ORF, including exons 2-5 and a portion of exon 6, was recovered. (C) Mapping of the breakpoints in the *Fak56^{CG1}* chromosome. The sequence alteration in the *Fak56^{CG1}* deletion mutant is flanked by the bases indicated. (D) The 5′ region of the *Fak56* gene is absent in *Fak56^{CG1}*. Genomic DNA from wild-type (1) and *Fak56^{CG1}* (2) flies were probed with FAKI and FAKII (covering 5′ and 3′ regions of the *Fak56* gene respectively), as well as Spt5 (the 5′ flanking gene) probes as indicated. Importantly, Southern analysis using the Spt5 probe indicates that this gene is intact in the *Fak56^{CG1}* mutant.

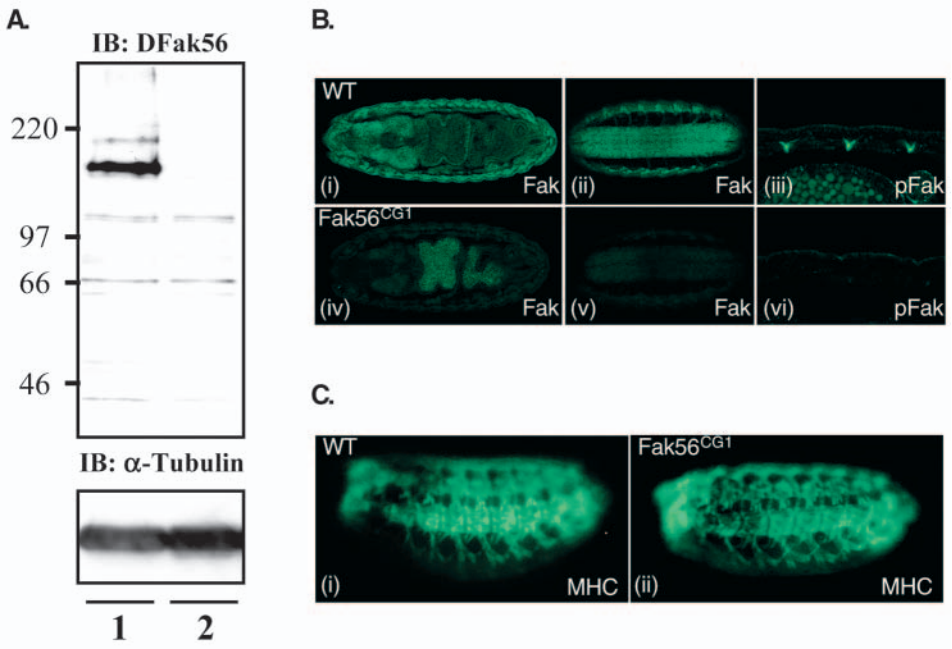

Fig. 2. Fak56 protein is not expressed in *Fak56*^{CG1} mutant animals. (A) Immunoblot analysis of wild-type (1) and *Fak56*^{CG1} (2) embryonic lysates using Fak56-specific antibodies, indicates that Fak56 protein is absent in *Fak56*^{CG1} mutant animals. Protein extracts were immunoblotted with anti-Fak56 antibodies (upper panel). Subsequently, the same blot was stripped and reprobed with anti-α-Tubulin antibodies to ensure equal loading. (B) Wild-type and *Fak56*^{CG1} embryos were analyzed with anti-Fak56 antibodies [(i), (ii), (iv) and (v)], as well as anti-phospho-FAK^{Y397} antibodies [(iii) and (vi)]. In wild-type embryos, Fak56 protein is ubiquitously expressed with particularly high levels in the embryonic CNS [(i) and (ii)]. In *Fak56*^{CG1} mutant embryos, Fak56 protein is absent [(iv) and (v)]. Similarly, in wild-type embryos, phosphorylated Fak56 was observed at muscle attachment sites (iii), and was strongly reduced in *Fak56*^{CG1} mutant embryos (vi). (C) Wild-type and *Fak56*^{CG1} embryos were analyzed with anti-myosin heavy chain (MHC) [(i) and (ii)]. No obvious muscle disruption phenotypes were observed in *Fak56*^{CG1} embryos when compared with wild-type. Embryo orientation is anterior to left and dorsal up.

recognized the endogenous Fak56 protein as a band of 140 kDa, which was completely absent from *Fak56*^{CG1} embryo extracts (Fig. 2A). Whole-mount staining of embryos with anti-Fak56 antibodies showed that the ubiquitous Fak56 stain observed in wild-type embryos was absent in the *Fak56*^{CG1} mutant embryos [Fig. 2B compare (i) and (ii), with (iv) and (v)]. Furthermore, using a phosphospecific anti-phospho-FAK^{Y397} antibody developed against mammalian FAK, which cross-reacts with Fak56 phosphorylated at the equivalent tyrosine 430 (see Figs S1, S2 in the supplementary material), we observed a strong localization of phosphorylated Fak56 at wild-type embryonic muscle attachment sites [Fig. 2B(iii)]. This was strongly reduced in *Fak56*^{CG1} mutant animals [Fig. 2B(vi)], consistent with these antibodies primarily recognizing phosphorylated Fak56. In summary, by DNA sequencing, Southern blotting, immunoblotting and immunostainings we conclude that *Fak56*^{CG1} is a null mutant allele of *Fak56*.

Having established that we had generated a null allele, we were surprised to find that the *Fak56*^{CG1} mutant animals are viable and fertile. Furthermore, no obvious developmental defects could be observed at any stage of development (see below). This suggested that the *Fak56* gene, and thus by extrapolation, this family of PTKs, is not of vital importance in *Drosophila*.

To make absolutely sure that we had completely inactivated Fak56 function we generated another deletion mutant that completely removes the *Fak56* gene as well as the adjacent *Calpain A* gene (Fig. 3A). This was achieved using the DrosDel isogenic deletion kit approach (Golic and Golic, 1996; Ryder et al., 2004). This deletion, named *Df(2R)ED3716*^{CG2}, was confirmed to be 12.8 kb in size and to have deleted *Fak56* by Southern, PCR and sequence analysis of the breakpoints (Fig. 3B,C). This deletion was found to be homozygous lethal, probably due to the loss of another gene, because: (1) the transheterozygotes *Fak56*^{CG1}*/Df(2R)ED3716*^{CG2} were null for Fak56 protein (see Fig. S3 in the supplementary material), and viable with no obvious phenotypes; and (2) the lethality of the larger deficiency was not rescued by a genomic *Fak56* transgene, even though it restores normal levels of Fak56 protein (see Fig. S3 in the supplementary material).

One of the most pronounced functions of integrins in the *Drosophila* embryo is to attach the somatic muscles to the epidermal tendon cells via an intervening tendon matrix (for reviews, see Bokel and Brown, 2002; Brower, 2003). Loss of integrins and associated molecules causes a muscle detachment or actin detachment phenotype and immobility of the mutant larva. While loss of Fak56 did not produce defects severe enough to cause lethality, it was possible that closer examination might reveal defects in integrin-related developmental processes. To investigate whether this was the case, we first examined the overall muscle pattern of *Fak56*^{CG1} mutant embryos by staining for muscle Myosin (MHC) and Actin (data not shown) and this revealed a normal patterning of the embryonic muscles in the absence of Fak56, as shown in Fig. 2C. We then investigated some key components of the integrin-containing adhesion complexes at the muscle attachment sites. However, the absence of Fak56 did not affect the localization of either subunit of the main muscle integrin, αPS2βPS, its ECM ligand Tiggrin, the cytoskeletal linker proteins Talin or Integrin-Linked Kinase (ILK), or the distribution of phosphotyrosine or Actin (Fig. 4 and Table S1 in the supplementary material), since all of these molecules showed a wild-type localization pattern in *Fak56*^{CG1} mutant animals.

We then confirmed these results with the larger lethal deficiency: *Df(2R)ED3716*^{CG2}. In agreement with our conclusions from the analysis of *Fak56*^{CG1} mutants, we found

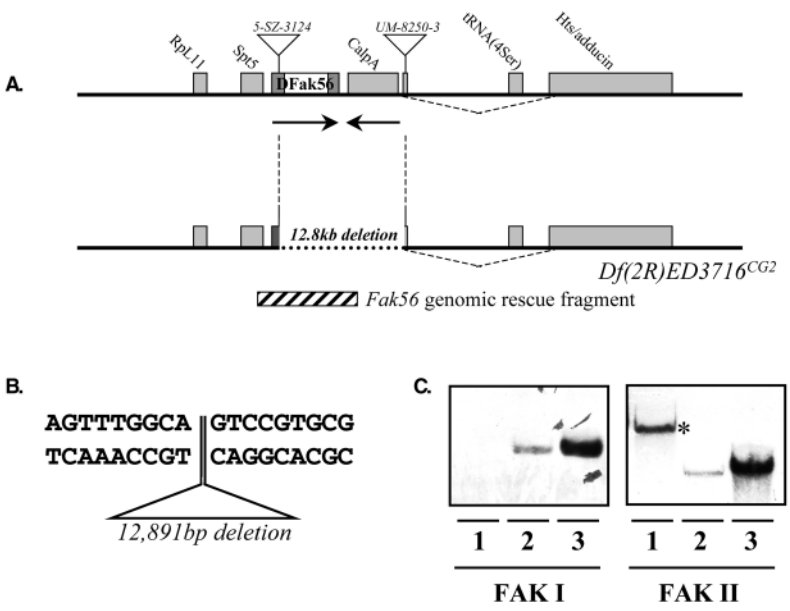

Fig. 3. Generation and characterization of the Df(2R)ED3716^{CG2} *Fak56* deletion mutant. (A) Genetic analysis of *Fak56*. Upper panel: the *Fak56* locus is depicted together with the surrounding genes (as before, Fig. 1). The two P-element insertions used to generate *Df(2R)ED3716*^{CG2}, *P(5-SZ-3124)* and *P(UM-8250-3)* are shown by inverted triangles. Lower panel: FRT recombination between the 5-SZ-3124 and UM-8250-3 P elements resulted in a 12.8 kb deletion, named *Df(2R)ED3716*^{CG2}, which uncovers both the entire *Fak56* ORF as well as the neighboring *Calpain A* gene. The *Fak56* genomic rescue fragment is also indicated (striped box). (B) Mapping of the breakpoints in the *Df(2R)ED3716*^{CG2} chromosome. The sequence flanking *Df(2R)ED3716*^{CG2} is shown. (C) Southern analysis of the *Df(2R)ED3716*^{CG2} mutant. The entire ORF of the *Fak56* gene is absent in *Df(2R)ED3716*^{CG2}. Genomic DNA from (i) *Df(2R)ED3716*^{CG2}*/Fak56* ^{CG1}, (ii) *Df(2R)ED3716*^{CG2}/+, and (3) wild-type flies were probed with FAKI and FAKII (covering 5′ and 3′ regions of the *Fak56* gene respectively) to show that the Fak56 ORF is completely absent in *Df(2R)ED3716*^{CG2} animals (* indicates the RFLP generated from the *Fak56*^{CG1} mutant).

that integrin localization and muscle attachment was also unaffected in *Df(2R)ED3716*^{CG2} mutant embryos (Fig. 5). We have thus been unable to find a role for Fak56 in the formation of the strong integrin adhesive junctions involved in muscle attachment. We therefore examined whether other integrin functions might require Fak56. Integrins are required for the normal migration of the primordial midgut cells (Martin-Bermudo et al., 1999; Roote and Zusman, 1995), and the transcriptional regulation in the midgut at later stages (Martin-Bermudo and Brown, 1999). However, in the absence of Fak56 integrin-regulated gene expression in the midgut, as well as migration of the primordial midgut cells appeared to occur normally (Fig. 4D).

The FAK family of PTKs has been implicated in multiple signaling transduction pathways (Gelman, 2003; Parsons, 2003). Therefore, we have examined the development of other embryonic tissues,

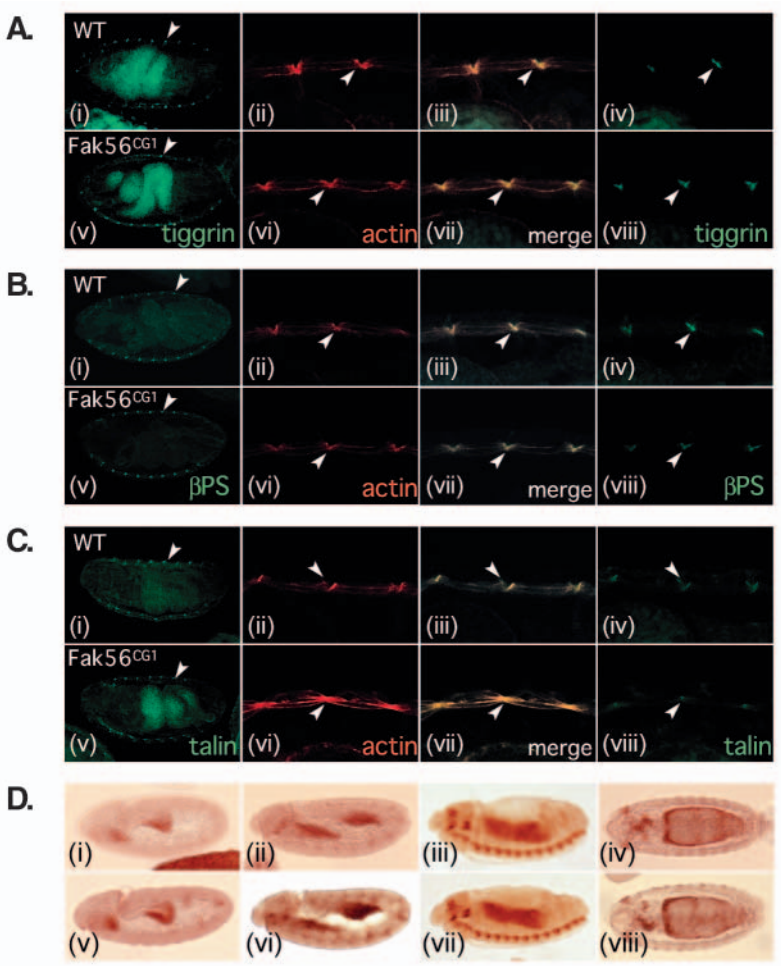

Fig. 4. Fak56 is not required for localization of components of adhesion complexes at muscle attachment sites. Wild-type and *Fak56*^{CG1} embryos were immunostained with antibodies recognizing proteins previously known to localize at muscle attachment sites. Three distinct protein components are shown, representing extracellular, transmembrane and intracellular components of the muscle attachment site. Wild-type and *Fak56*^{CG1} embryos were stained with anti-Tiggrin (A), anti-βPS integrin (B), and anti-Talin (C) antibodies (green). Both genotypes showed wild-type localization at muscle attachment sites (arrowheads). All embryos were double-stained with Rhodamine-Phalloidin (red) to visualize actin filaments and muscle attachment sites. Low magnification is shown in (i) and (v). High magnifications of the muscle attachment sites are shown in (ii)-(iv) and (vi)-(viii). (D) Fak56 is not required for migration of the primordial midgut cells. The enhancer trap insertion line 258 was used to study the migration of the primordial midgut cells in Fak56 mutant embryos. The endodermal midgut arises from two primordia, the anterior midgut (AMG) and the posterior migut (PMG) primordium [(i) and (v), stage 10 embryos]. To form the midgut, these extend toward each other during stages 11 and 13 [(ii), (iii), (vi) and (vii)] and fuse laterally on both sides of the yolk, at stage 14 [(iv) and (viii)]. No obvious defects in the migration of primordial midgut cells could be observed in *Fak56*^{CG1} mutant embryos, when compared with wild-type controls.

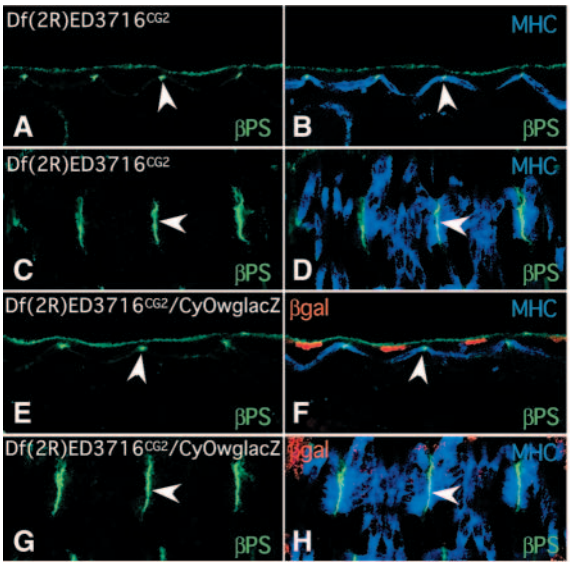

Fig. 5. Muscle attachments sites are unaffected in *Df(2R)ED3716^{CG2}* mutant embryos. Muscle attachment sites were analyzed in *Df(2R)ED3716^{CG2}* mutants. *Df(2R)ED3716^{CG2}* mutant (A-D), and control *Df(2R)ED3716^{CG2}/CyOwglacZ* embryos (E-H) were stained with anti-βPS integrin antibodies to visualize muscle attachment sites (A,C,E,G). Embryos were double-stained with anti-myosin heavy chain (MHC) to visualize gross muscle structure (B,D,F,H). Both genotypes showed wild-type localization of βPS integrin at muscle attachment sites (arrowheads), as well as normal muscle morphology. Mutant embryos were identified by absence of lacZ balancer (red in F and H).

including the tracheal system (using 2A12), the CNS (using 22C10 and Elav), the salivary glands (using anti-CREB) and the visceral mesoderm (using anti-ALK and anti-FasIII). In every case the development of these tissues was normal, as the pattern of staining was indistinguishable from the wild-type control embryos (see Table S2 in the supplementary material). Particularly high levels of Fak56 protein have been found during two developmental events in *Drosophila*, the migration of a subset of follicle cells, the border cells, during oogenesis, and in the development of the gonad (Bai et al., 2000; Cohen et al., 2002; Fox et al., 1999). We examined these processes, but again found no pertubation in the absence of Fak56 (see Figs S4, S5 in the supplementary material). In addition, careful examination of the developing tracheal system revealed no defects in the migration of this developing tissue (see Fig. S6 in the supplementary material).

Despite the fact that removing Fak56 does not have severe consequences, overexpressing this protein clearly does. We have seen from earlier experiments that overexpression of Fak56 results in multiple phenotypes. This together with the intriguing localization of phosphorylated Fak56 protein at muscle attachment sites (Fig. 2) led us to investigate further the effect of Fak56 function using the UAS-GAL4 system (Brand and Perrimon, 1993) to drive expression in a muscle-specific fashion. In order to do this we employed Mef2-Gal4, which results in expression specifically in the muscles. Overexpression of Fak56 resulted in a potent muscle detachment phenotype (Fig. 6D-F). This phenotype was indistinguishable in severity to that caused by the absence of integrins in the muscles, due to the lack of αPS2 (Brown, 1994), and in both cases development appeared to occur normally prior to detachment. Moreover, we could observe that in spite of the obvious rounding up of somatic muscles, integrins, as monitored by anti-β-integrin antibodies (Fig. 6A,D), could still be found at the ends of the muscles that remain attached.

We next examined the effects of Fak56 overexpression in greater detail, employing both Fak56 and Fak56-GFP fusion proteins, in an effort to elucidate a mechanism behind the muscle detachment phenotype. Initially we noticed that Fak56-GFP itself is strongly targeted to muscle attachment sites upon overexpression (Fig. 7C,F), and becomes autophosphorylated (see Fig. S2 in the supplementary material). This may be due to the FAT homology domain present in the C-terminal of Fak56, which has been shown to be responsible for the targeting of mammalian FAK to focal adhesions (Hildebrand et al., 1993; Hildebrand et al., 1995). We then examined the effects of Fak56 or Fak56-GFP overexpression on a number of ECM and cytoskeletal proteins involved in adhesion at muscle attachment sites. Overexpression of Fak56 causes loss of muscle adhesion without affecting the initial localization of αPS2 integrin. However, in muscles that detach, we observed that αPS2 integrin remains at the ends of the detached muscles indicating its dissociation from the extracellular matrix (Fig. 7A,B). Direct immunolocalization of αPS2 and the ECM ligand Tiggrin further confirmed this observation, since we did not find particular enrichment of Tiggrin at the ends of the detached muscle (Fig. 7M-P). This intriguing result suggests that excess Fak56 at the site of integrin adhesion to the ECM may result in a changed affinity of the integrin for ECM components and a subsequent release of the muscle membrane at the attachment site itself. Localization of ECM ligands, such

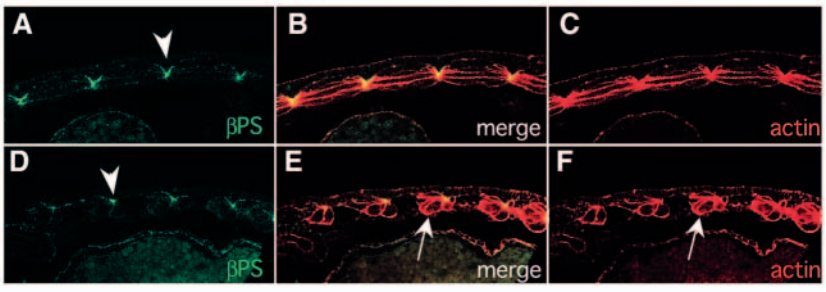

Fig. 6. Overexpression of Fak56 results in embryonic muscle detachment. Wild-type embryos and embryos expressing Fak56 specifically in embryonic muscle, using the Mef2-GAL4 driver, were stained with anti-βPS antibodies (green) to visualize integrins and Phalloidin (red) to visualize actin. Embryos overexpressing Fak56 specifically in embryonic muscle, show a potent muscle detachment phenotype (E,F, arrows), when compared with wild-type (B,C). Interestingly, in spite of obvious rounding up of somatic muscles, integrins, as monitored by anti-βPS-integrin antibodies, were still localized at the ends of the muscles that remain attached in Fak56 overexpressing embryos (D, arrowhead), indistinguishable to wild-type embryos (A, arrowhead).

as Tiggrin (Fig. 7I,L) or wing blister (laminin $\alpha_{1,2}$) (data not shown), showed no abnormal distribution as a result of Fak56 overexpression, suggesting that the integrin detachment we see is not due to a simple defect in the assembly of the ECM itself. Furthermore, we examined the localization of two intracellular components essential for the link to actin filaments, Talin (Fig. 7D,E) and ILK (Fig. 7J,K), and found that their localization at muscle attachment sites was unaffected by Fak56 overexpression. Thus, we conclude that the presence of excess

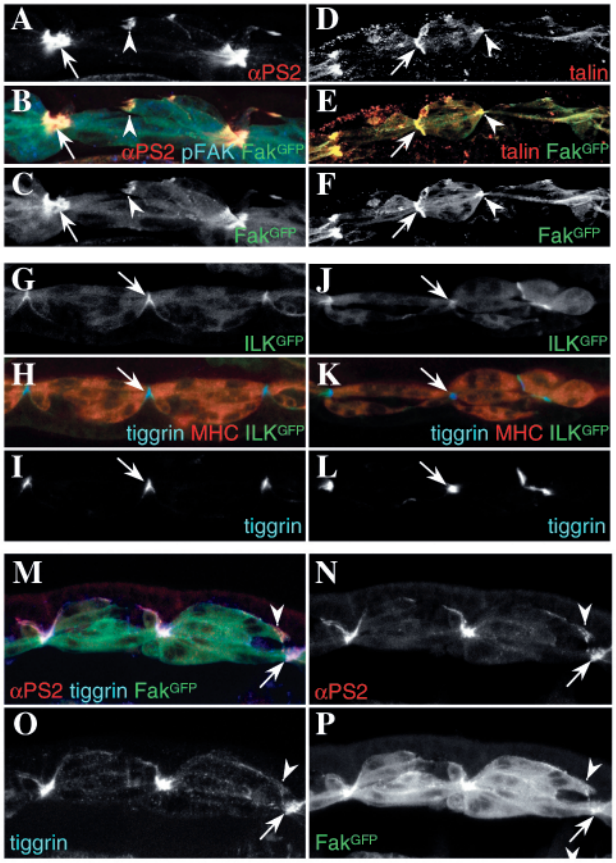

Fig. 7. Overexpression of Fak56 downregulates integrin adhesion to the extracellular matrix without affecting linkage to the cytoskeleton. Muscles and their attachment sites in the epidermis are visualized in stage 16 embryos expressing either Fak56-GFP (A-F,M-P) or Fak56 (J-L) specifically in embryonic muscle, using the Mef2-GAL4 driver, or control embryos (G-I). (A-F) Embryos overexpressing Fak56-GFP specifically in muscle were stained with antibodies against αPS2 integrin (red) (A,B), or Talin (red) (D,E), or antiphospho-FAKPY397 (blue) (B). Fak56-GFP is localized at the muscle ends (C,F, arrows), and its overexpression causes loss of muscle adhesion, without affecting the initial localization of αPS2 integrin. However in muscles that detach, αPS2 integrin remains at the ends of the detached muscles (A,B, arrowheads) indicating its dissociation from the extracellular matrix. A similar effect on Talin localization is observed (D,E, arrowheads). (G-L) Embryos expressing ILK-GFP are shown probed with anti-myosin heavy chain (MHC, red), and the extracellular matrix component Tiggrin (blue) (I,L). In embryos overexpressing Fak56 (J-L), ILK-GFP and Tiggrin are localized at the muscle ends similar to controls (G-I). Embryos expressing Fak56-GFP analyzed with anti-αPS2 (red) and Tiggrin (blue) (M-P). In embryos overexpressing Fak56-GFP, Tiggrin can be seen at the attachment site (arrows), but is not particularly enriched at the ends of the detached muscles (arrowheads).

Fak56, and thus excess Fak56 PTK activity, does not appear to result in the disassembly of the integrin-actin link, but rather appears to result in a detachment of the plasma membrane via dissociation of the integrins themselves from the ECM. These results suggest that Fak56 overexpression may result in the negative modulation of integrin ligand binding affinity.

It is clear that the overexpression of Fak56 causes more severe defects than the loss of Fak56 itself, thus indicating that it is not acting simply as a dominant negative on endogenous Fak56, but instead is inhibiting other processes. This effect does not appear to be due to a general problem with excess tyrosine phosphorylation, since overexpression in the epidermis did not cause any defects, despite Fak56 being expressed at high levels and being activated by autophosphorylation (see Fig. S2 in the supplementary material). The reduction in integrin affinity for the ECM might be important for migration, and therefore we tested whether overexpression of FAK caused an acceleration of the rate of primordial midgut migration, but could not detect a significant change (data not shown). In the developing wing epithelium, Talin has been found to be essential for the formation of focal contact-like structures on the basal suface (Brown et al., 2002). This contrasts with the muscles, where loss of Talin does not affect integrin concentration to the ends of the muscles, but is required for the link of integrins to the cytoskeleton. Overexpression of Fak56-GFP in the wing epithelia perturbed the appearance of the focal adhesion structures, but they still formed and contained overexpressed, phosphorylated Fak56 (see Fig. S7 in the supplementary material).

As Fak56 overexpression results in a loss of integrin phenotype, we wished to confirm in another way that this was directly inhibiting integrin function. If this were the case, then reducing the amount of integrins should enhance the phenotype caused by Fak56 overexpression. Using the Eng-Gal4 driver, which drives expression in the posterior portion of the wing, we have previously shown that overexpression of Fak56 induces wing blistering (Palmer et al., 1999). Null mutants of the gene encoding the βPS integrin subunit, *myospheroid* (*mys*), are lethal; however, hypomorphic alleles exist that are homozygous viable. In order to ask experimentally whether the wing blistering we observe on overexpression of Fak56 is sensitive to mutations in the β-integrin subunit, we employed two different alleles, mys^{nj42} (Wilcox et al., 1989) and mys^{b43} (Jannuzi et al., 2004). In control flies, at 25°C, overexpression of Fak56 in the wing resulted in wing blisters in 28% of adult females (Fig. 8B). Under these conditions, 0% of male flies displayed wing blisters, although minor wing defects could be observed [Fig. 8A(ii),B]. In a background of either mys^{nj42} or mys^{b43} an enhancement of wing blistering was observed in adult females – to 44% and 40% respectively (in this case females are heterozygous for the respective *mys* allele) (Fig. 8B). However, when we observed adult males, which were hemizygous for the *mys* alleles, we observed a dramatic increase in wing blistering – from 0% to 31% (for mys^{nj42}) and 21% (for mys^{b43}) [Fig. 8A(iii) and B]. Thus, this confirms that overexpressed Fak56 acts negatively on integrin adhesion in *Drosophila*.

The localization of phosphorylated Fak56 protein at muscle attachment sites in wild-type embryos (Fig. 2B), together with the strong muscle detachment phenotype observed upon the overexpression of Fak56 in the muscles themselves (Figs 6, 7)

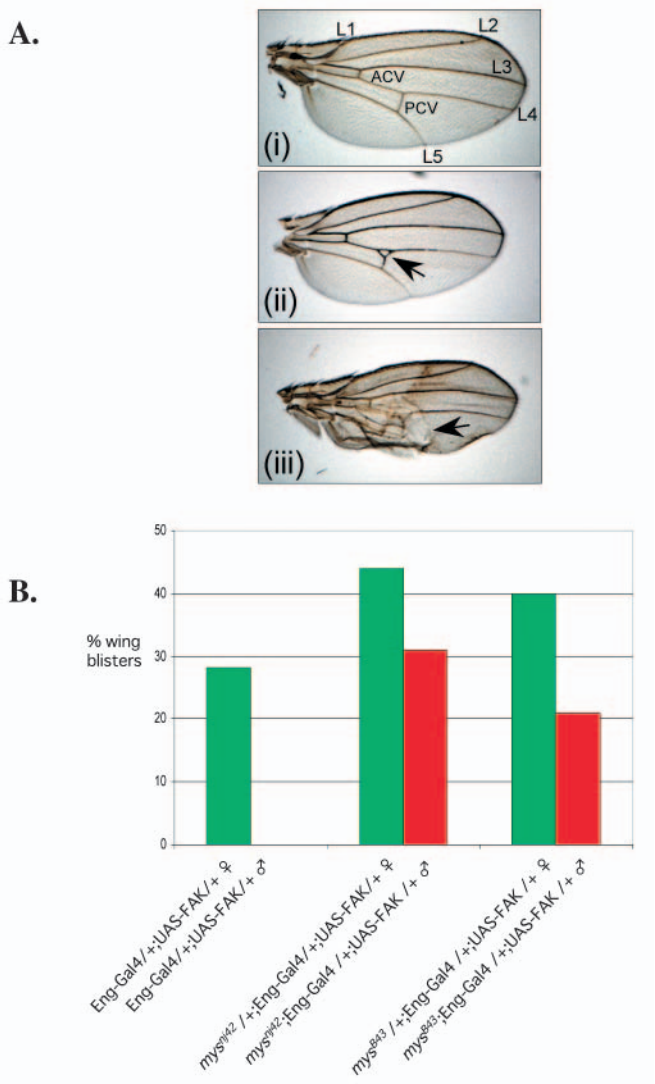

Fig. 8. Fak56 and βmys-integrin interact genetically in the *Drosophila* wing. Wing blister formation induced by expression of Fak56 in the developing wing disc is sensitized in an integrin mutant background. (A) Light micrographs of wings overexpressing Fak56. A(i) Engrailed-*GAL4/+* control showing wild-type wing morphology. Wing veins L1-5, anterior cross vein (ACV) and posterior wing vein (PCV) are indicated. A(ii) *Engrailed-GAL4/+;UAS-Fak56/+* male, A(iii) βmys^{b43};*Engrailed-GAL4/+;UAS-Fak56/+* male wings are shown. Arrows indicate wing defects or blisters. (B) Quantification of the wing blistering observed for the genotypes: *Engrailed-GAL4/+;UAS-Fak56/+* female (n=241) and male (n=172), βmys^{nj42};*Engrailed-GAL4/+;UAS-Fak56/+* female (n=246) and male (n=117) and βmys^{b43};*Engrailed-GAL4/+;UAS-Fak56/+* female (n=160) and male (n=154) is shown. Numbers in brackets after the genotype indicate the number of flies scored.

implies, that while not critical, Fak56 does play some kind of accessory role in integrin-mediated adhesion processes. In order to investigate this further we decided to look at Fak56 in integrin mutants themselves. Analysis of $inflated^{B4}$ mutant embryos using phospho-FAK^{Y397} antibodies, revealed that there is a complete loss of phosphorylated Fak56 at the muscle attachment sites (Fig. 9B), when compared with wild-type (Fig. 9A). Thus, Fak56 does appear to be activated at the

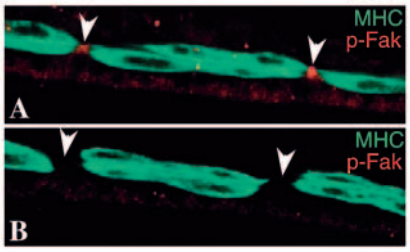

Fig. 9. Tyrosine phosphorylation of Fak56 at embryonic muscle attachment sites is integrin dependent. Wild-type and $inflated^{B4}$ mutant embryos were stained with anti-muscle myosin heavy chain (MHC) (green) and anti-phospho-FAK^{Y397} antibodies (red). (A) In wild-type embryos phosphorylated Fak56 is localized at muscle attachment sites (arrowheads), whereas in $inflated^{B4}$ mutants no phosphorylated Fak56 can be observed (B).

muscle attachment site in an integrin-dependent manner, in agreement with earlier experiments where plating of primary *Drosophila* embryo cultures on extracellular matrix components such as Tiggrin and Laminin results in the tyrosine phosphorylation of Fak56 (Palmer et al., 1999). However, while integrin-mediated attachment appears to involve the activation of Fak56, the lack of defects in either integrin signaling or integrin-mediated adhesion in *Fak56* mutant embryos implies that activation of, and indeed the presence of, Fak56 is clearly not essential for these processes.

Discussion

In this paper we report the surprising finding that the sole *Drosophila* FAK family member, Fak56, is not required for integrin-mediated developmental functions in vivo. Moreover, Fak56 function is not essential for overall viability in *Drosophila*, since the *Fak56* mutant flies we have generated are both viable and fertile. From our extensive investigations we are convinced that Fak56 is not required for the proper development of multiple embryonic tissues, including somatic musculature, the tracheal system, gonads, salivary glands or gut. Most surprisingly, we have observed that Fak56 does not seem to be essential for either muscle attachment or the proper localization of components of integrin adhesive sites to muscle attachment sites. Since no other FAK family PTK exists in the *Drosophila* genome, we have no option other than to conclude that FAK family PTKs are not required, and thus are not essential players in integrin function in vivo. While this conclusion may be controversial, data from *C. elegans* indicate that FAK is also non-essential in the nematode. Again, there appears to be only one FAK family member in the genome of this model organism (Harris et al., 2004; Harris et al., 2003; Stein et al., 2001), thus eliminating the possibility of redundancy explaining these results. While this conclusion has not been formally reported for *C. elegans*, two separate findings support this hypothesis: (1) RNAi towards FAK (known as kin-32 in the nematode) causes no obvious phenotypes; and, in addition, (2) a large deletion in the kin-32 open reading frame is viable (data from Wormbase website, http://www.wormbase.org) (Harris et al., 2004; Harris et al., 2003; Stein et al., 2001).

In *Drosophila*, mutants for many of the proteins known to

localize at the muscle attachment site cause lethality, in many cases due to muscle detachment, i.e. the integrins themselves (reviewed by Bokel and Brown, 2002; Brower, 2003), ILK (Zervas et al., 2001), Talin (Brown et al., 2002), Tiggrin (although 1% of flies eclose) (Bunch et al., 1998), Laminin (Henchcliffe et al., 1993), α-actinin (Fyrberg et al., 1990), and PINCH (Clark et al., 2003). Other molecules, such as the *Drosophila* Tensin homologue (also known as Blistery), are not lethal, but do display phenotypes indicative of failure of adhesion, such as wing blistering (Lee et al., 2003; Torgler et al., 2004). However, it is interesting to note that another cytoskeletal protein – Vinculin – has also been shown to be non-essential in *Drosophila* (Alatortsev et al., 1997), in contrast with previous results in the mouse, where animals mutant for the *vinculin* gene display a lethal phenotype due to heart and brain defects during embryogenesis (Xu et al., 1998). The non-essential nature of Fak56 in *Drosophila* perhaps explains why extensive attempts to target *Fak56* through various methods have thus far been unsuccessful, since the general assumption has been that such mutants would be lethal. Moreover, this may also be the reason why, despite extensive genetic screening by several groups with the purpose of identifying molecules involved in integrin-mediated signaling, no mutations have ever been identified in *Fak56* (C.G. and R.H.P., unpublished), although the effectiveness of these screens has been demonstrated by the fact that they have independently identified several common loci in addition to existing PS integrin genes (Prout et al., 1997; Walsh and Brown, 1998).

While Fak56 protein appears to be ubiquitously expressed (Fox et al., 1999; Fujimoto et al., 1999; Palmer et al., 1999), phospho-Fak56 is strongly localized at muscle attachment sites. This implies that Fak56 is not only localized, but also activated at these locations, since phosphorylation of the FAK^{Y397} site (which is conserved in Fak56), is considered to reflect FAK activation in vivo (Calalb et al., 1995). The anti-phospho-FAK^{Y397} antibodies seem to be specific for phosphorylated *Drosophila* Fak56, based on two criteria: (1) loss of immunoreactivity in the *Fak56* mutants, and (2) overexpressed wild-type Fak56 is recognized by the antiphospho-FAK^{Y397} antibodies, whereas overexpressed $Fak56^{Y430F}$ mutant protein is not (data not shown). A role for Fak56, albeit an accessory one, at muscle attachment sites, is endorsed by our finding that phosphorylated Fak56 is absent from muscle attachment sites in integrin mutants. Thus, while not required for integrin function, Fak56 appears to be involved through an as yet undefined mechanism in integrin-mediated events in *Drosophila* embryogenesis. In spite of the absence of the Fak56 PTK at muscle attachment sites in *Fak56* mutant embryos, the levels of phosphotyrosine observed are indistinguishable from wild-type (Table S1 in the supplementary material), suggesting that another PTK(s) is activated at these sites. This is interesting in light of a recent report that Src kinases can be activated by direct interaction with integrin β-subunit tails (Arias-Salgado et al., 2003). Such a model implies that Src family kinases can be activated by integrins independently of FAK and could explain the unexpected lack of phenotypes in the *Fak56* mutant. Interestingly, it has recently been shown that v-Src transformation of *Ptk2*$^{-/-}$ fibroblasts rescues the motility defects observed in *Ptk2*$^{-/-}$ fibroblasts, which is in keeping with these results (Hsia et al., 2003; Moissoglu and Gelman, 2003).

Consistent with an accessory involvement of FAK in integrin-mediated adhesion, we show here that the Fak56-induced wing blister phenotype is sensitized in an integrin mutant background, thus indicating a genetic interaction between Fak56 and integrins in this context. Employing the UAS-GAL4 system to drive Fak56 specifically in muscles it is clear from our results that overexpression of Fak56 disrupts muscle attachment, thus overexpression of Fak56 causes much more serious defects than its absence. This is consistent with Fak56 either functioning as an adaptor by displacing more critical proteins from the integrin cytoplasmic tail required for the extracellular binding to ECM ligands, or causing a dissociation of the integrin-containing complex by excessive phosphorylation. The evidence in mammalian systems suggests that FAK plays a critical role in cell migration, which is a complex, highly regulated process that involves the continuous formation and disassembly of adhesions (Gelman, 2003; Parsons, 2003; Schlaepfer and Mitra, 2004). Indeed, recently it has elegantly been shown that FAK is important for adhesion turnover at the cell front, a process central to migration (Webb et al., 2004). We have examined the migration of (1) the endoderm, (2) border cells during oogenesis, (3) germ cells and (4) the trachea, in *Fak56* mutant animals, and have found no defects in either process in the absence of Fak56. However, such a role of FAK in the disassembly, rather than in the actual assembly of these structures, is in agreement with our findings that Fak56 overexpression results in detached muscles where the αPS2 integrin is still localized at the muscle ends, as though dissociated from the ECM. This then raises the question of why Fak56 is normally found in the developing muscles. We speculate that it contributes in keeping the strong adhesive junctions dynamic so that they can be remodeled during the formation of the attachments and the subsequent growth of the muscles. Perhaps a defect in this process would be more apparent under more strenuous growth conditions than those found in the laboratory.

One important point to consider is the wealth of data from mammalian cell experiments indicating a critical role for FAK family PTKs in integrin function (reviewed by Gelman, 2003; Parsons, 2003). Such a function is corroborated by the phenotype of the FAK knockout mouse, which dies early in embryogenesis (Ilic et al., 1995). Indeed, *Ptk2*$^{-/-}$ fibroblasts derived from FAK mutant embryos, exhibit a rounded morphology, and have an increased number of focal contact sites and decreased rates of cell migration (Ilic et al., 1995). A recent RNAi-based screen of the *Drosophila* genome to find novel genes affecting cell morphology identified Fak56 as a molecule affecting cell spreading (Kiger et al., 2003). In addition, overexpression of Fak56 in the fly using the GAL4-UAS system also produces a wealth of integrin-related phenotypes (C.G. and R.H.P., unpublished). We have noted that overexpressed Fak56 does localize to muscle attachment sites under such conditions, and it is entirely possible that this creates a neomorphic phenotype, i.e. such that the presence of excess Fak56 protein – or indeed Fak56 activity – binds up multiple factors and acts in a dominant negative fashion to produce these defects. Thus, experiments in mammalian cells employing overexpression of FAK to analyze integrin function may not be an ideal model for the in vivo scenario.

Furthermore, a radical explanation for the strong defects observed in *Ptk2*$^{-/-}$ fibroblasts, which would be consistent with our findings, is if the primary defect of removing FAK is the observed elevated expression and activity of the related kinase Pyk2, and the Src family PTKs (Sieg et al., 1998). If overexpressed Pyk2 causes similar dominant negative effects on other components of integrin adhesion, as we have seen when Fak56 is overexpressed, than this could contribute to the severe phenotype observed in the FAK mutant mice. Finally, it is also possible that the functions of the FAK family PTKs are not totally conserved between *Drosophila* and mice, and that FAK has assumed a more critical role during evolution of vertebrates.

Disruption of the single *Drosophila Fak56* gene is the first example of an animal completely lacking FAK PTK function. However, it is surprising that a genetic null for a protein such as FAK, that has always been thought to be a critical link between integrins and the actin cytoskeleton, exhibits a viable phenotype. Since this also appears to be true for the *C. elegans* FAK family PTK, we must assume that while FAK may play an accessory role in modulating integrin functions in vivo, it is by no means essential for integrin-mediated adhesion or signaling in the fruitfly. It is obvious that further experiments will be required to define and understand the exact role of the Fak56 PTK in such processes in vivo.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/131/23/5795/DC1

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Table S1. Localisation of muscle attachment site proteins in Fak56 mutant embryos

Muscle attachment site proteins analysed in this study	Localisation in Fak56^{CG1}
Fak56	Absent
Fak56-PY397(430)	Absent
Phosphotyrosine	Wild type
βPS integrin	Wild type
αPS2 integrin	Wild type
Muscle myosin (MHC)	Wild type
Actin (Phalloidin)	Wild type
Talin	Wild type
Tiggrin	Wild type
ILK-GFP	Wild type

Table S2. Analysis of various tissue development in Fak56 mutants

Other markers used to analyse Fak56^{CG1}	Localisation in Fak56^{CG1}
Fasciclin 3	Wild type
β-Tubulin	Wild type
22C10 (CNS)	Wild type
Elav	Wild type
2A12 (Trachea)	Wild type
ALK (Visceral mesoderm)	Wild type
CREB (Salivary glands)	Wild type
Vasa (Germ cells)	Wild type
258-*lacZ* (Endoderm)	Wild type

Direct immunohistochemical analysis of Fak56 mutants was carried out with antibodies directed against the proteins indicated. The developing central nervous system was examined with mAb22C10 and anti-Elav, the tracheal system with 2A12, salivary gland migration with anti-CREB, the visceral mesoderm with anti-DAlk and Fas3, the germ cells with anti-Vasa, and the endoderm with 258-*lacZ*.

Data S1. Additional materials and methods

Immunostainings and antibodies

Mouse monoclonal anti-GFP antibody was used at 1:1000 (Clontech), mouse monoclonal anti-PY100 antibodies were used at 1:1000 (Cell Signalling), rabbit anti-β-gal antibodies were used at 1:150 (ICN Biomedicals), rabbit anti-Vasa antibodies (Lasko and Ashburner, 1990) were used at 1:500 (a kind gift from P. Lasko). Mouse monoclonal 2A12 antibody, which recognises an unknown tracheal luminal component was used at 1:5 (Developmental Hybridoma Bank). Anti-CREB antibodies (Andrew et al., 1997) were used at 1:15,000 (kind gift from D. Andrew), guinea pig anti-ALK antibody was used at 1:1000, mouse monoclonal anti-Fas3 antibodies were used at 1:50, mouse monoclonal anti-Elav (958A9) was used at 1:1000 and mouse monoclonal 22C10 was used at 1:50 (Developmental Hybridoma Bank). DAPI was used at a final concentration of 0.5 g/ml.

Generation of Fak56 transgenic flies

The pBluescript:Fak56 (Palmer et al., 1999) was used as a template to create the Fak56-GFP fusion protein. A C-terminal segment of Fak56, corresponding to amino acids 1121-1204, was amplified by standard PCR using forward (5′-TTCTGGAGATTTGCTTCA-3′) and reverse (5′-TGCTCGAGCACTGTGCGGTAACTGTG-3′) primers. The resulting PCR fragment was digested with *Bsa*BI/*Bam*HI, and subcloned in frame with GFP in the pEGFP vector (Clonetech) (digested with *Bsa*BI/*Bam*HI), to create pEGFP:Fak56 aa1135-1204-GFP. Subsequently, pEGFP:Fak56 aa1135-1204-GFP was digested with *Mun*I/*Xba* I and the 964 bp fragment, encoding Fak56 aa1135-1204-GFP, subcloned back into the vector encoding full-length Fak56 (pBluescript:Fak56) (digested with *Mun*I/*Xba*I). This construct, named pBluescript:Fak56-GFP was confirmed by sequence analysis. To create pUAST:Fak56-GFP, pBluescript:Fak56-GFP was digested with *Xho*I/*Xba* I and the resulting 4434 bp fragment subcloned into pUAST vector (digested with *Xho*I/*Xba*I). The *Fak56* genomic rescue transgene was generated by subcloning a 10,189bp *Apa*I-*Alw*NI genomic DNA fragment containing 1,883 bp upstream of the nucleotide corresponding to the initiation AUG codon in the *Fak56* gene, and 2795 bp downstream of the polyA site, into the P-element transformation vector pCasper4. P element transformation was performed by microinjection together with a delta 2.3 transposase source containing plasmid, into $white^{1118}$ embryos.

Immunostainings of ovarioles

Ovaries from Fak56 mutant and wild-type fattened flies were dissected in PBS, fixed in PBS containing 4% formaldehyde for 20 minutes, rinsed, and blocked in NP40 block buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% NP40, 5 mg/ml BSA) overnight at 4°C (Bai et al., 2000). Egg chambers were incubated with rhodamine-phalloidin (Molecular Probes) and DAPI at a final concentration of 0.5 g/ml for 1 hour at room temperature. After three washes, egg chambers were mounted in PVA/DABCO mounting solution and analysed by confocal microscopy.